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14. ABSTRACT

This report describes the seventh year of research, and the second under this award, by a Consortium of investigators who have been continuously funded by this Program to develop, characterize, and utilize strains of mice that accurately model tumors found in persons with NF1 and NF2. This Consortium has generated many novel models of NF1 and NF2-associated tumors and has exploited these strains to investigate biologic and preclinical questions. These strains are a cornerstone of a recent initiative by the Children's Tumor Foundation to organize and support a preclinical network for testing therapeutics that might benefit persons with NF1 and NF2 disease. The investigators collaborate closely and share expertise and reagents extensively. This NF Consortium is a member of the Mouse Models of Human Cancer Consortium of the National Cancer Institute and is participating fully in the activities of the group.

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INTRODUCTION

Benign and malignant tumors are a major cause of morbidity and mortality in individuals with NF1 and NF2. The *NF1* and *NF2* genes function as tumor suppressors in humans and mice. Although a great deal has been learned about the genetics, biochemistry, and cell biology of NF1 and NF2-associated tumors, it has proven difficult to translate these advances into new treatments. The development of accurate, well-characterized mouse models of NF-associated tumors NF1 and NF2 represent an invaluable resource for bringing improved treatments to NF patients. The overall purpose of this consortium, which has been in existence for 7 years, is to develop such models so that they will serve as permanent resources for the scientific community. These efforts are timely for a number of reasons.

First, advances in gene targeting technologies have made it feasible to introduce many types of alterations into the mouse germline. Indeed, investigators who pioneered this general strategy were awarded the Nobel Prize in Medicine in 2007. The members of this research consortium developed the initial strains of *Nf1* and *Nf2* mutant mice, which provided major insights into a number of the complications seen in human NF1 and NF2 patients. Since the inception of this consortium effort in 2000, we have made substantial progress by engineering conditional mutant alleles of both *Nf1* and *Nf2* and in using these to create tractable new models for biologic and preclinical studies. Second, investigating cells from these *Nf1* and *Nf2* mutant mice has provided numerous fundamental insights that are directly relevant to understanding deregulated growth in *NF1* and *NF2*-deficient human cells. Genetic analysis of human and murine tumors has provided compelling evidence that *NF1/Nf1* and *NF2/Nf2* function as tumor suppressor genes (TSGs) *in vivo.* Biochemical data have suggested target proteins and pathways for rational drug design. The improved mouse models developed by this consortium also provide invaluable platforms for rigorous preclinical trials of these innovative approaches. Third, new therapies are urgently needed for many of the tumors that arise in individuals with NF1 and NF2. The current treatments for neurofibroma, optic nerve glioma, vestibular schwannoma, and for NF1 and NF2-asociated malignancies are frequently ineffective and carry a substantial risks of long-term morbidities. This consortium is highly complementary to the ongoing efforts of the NF Clinical Trials Consortium that is supported by this Program, which involve investigating novel treatments in NF patients, as the models that we have created facilitate testing novel agents and approaches in a controlled preclinical setting. These models are also integral to a new initiative of the Children's Tumor Foundation to organize and support a preclinical network for testing therapeutics that might benefit persons with NF1 and NF2 disease that may lead to innocative trials by the NF Clinical Trials Consortium. The quantity of drug required, expense, time to obtain data, and potential liability are all either greatly reduced or eliminated in mouse models. These strains therefore facilitate testing a wide range of new therapies that might benefit NF patients. Finally, the Mouse Models of Human Cancer Consortium (MMHCC) of the National Cancer Institute (NCI) is providing an opportunity for interactions among ~20 research groups that are working to develop, validate, and enhance models of a variety of human cancers. NF is the only inherited cancer predisposition represented within the MMHCC as a discrete disease entity. Our group was admitted to the MMHCC in 2000 and has been participating in its activities. Drs. Jacks, Parada, and Shannon are members of the MMHCC Steering Committee, with Dr. Parada serving as the designated representative of the NF Consortium. Dr. Jacks was Co-Chair of the Steering Committee from the inception of the MMHCC until 2002, and Dr. Shannon served as Co-Chair from 2002-2005. Thus, this award has provided the NF research

community with an exceptional level of representation within the mouse cancer modeling community. The MMHCC has spearheaded efforts in areas such as building repositories, devising pathologic classification schemes, imaging mouse tumors, and stimulating interactions with industry in the area of preclinical therapeutics that are of general importance to NF research. Our work under this award focuses on the two overall technical objectives (aims) listed below.

(1) To enhance existing strains of *Nf1* and *Nf2* mutant mice and to develop new *in vivo* models of NF-associated tumors. We will fully characterize lesions that arise in these mice, focusing on how closely they reproduce the phenotypic, genetic, and biochemical alterations seen in comparable human tumors.

(2) To perform *in vitro* and *in vivo* studies to elucidate biochemical pathways underlying the proliferative advantage of *Nf1* and *Nf2*-deficient cells as a way of identifying molecular targets for therapeutic interventions.

These technical objectives included a series of studies that were organized under specific subheadings in our application. For clarity, this Progress Report follows a similar format.

BODY

Background

Tumor Spectrum in NF1 and NF2 Patients. Persons with NF1 are predisposed to benign neurofibromas, optic nerve gliomas, and to specific malignant neoplasms. Individuals with NF1 typically develop multiple neurofibromas that can result in cosmetic, orthopedic, and neurologic disabilities. Optic nerve gliomas are another vexing clinical problem. Although histologically benign, these tumors frequently cause visual impairment or blindness because of their anatomic location. The malignant neoplasms seen in NF1 patients include astrocytoma, malignant peripheral nerve sheath tumor (MPNST), pheochromocytoma, and juvenile myelomonocytic leukemia (JMML). NF2 affects 1 in 40,000 persons worldwide. NF2 affects 1 in 40,000 persons worldwide. Individuals with NF2 develop schwannomas along cranial nerves (especially the eighth nerve), as well as peripheral nerves. Other NF2-related tumors include meningiomas, gliomas, and ependymomas.

Production and Characterization of *Nf1* and *Nf2* Mutant Mice. Drs. Jacks and Parada independently disrupted *Nf1* by inserting a neomycin *(neo)* cassette into exon 31 (1, 2). Homozygous *Nf1* mutant ($Nf1^{-/-}$) embryos die *in utero* with cardiac anomalies, which precludes the use of these mice to study important aspects of NF1 pathology, including the formation of many tumor types. To circumvent this problem, Dr. Parada's lab harnessed *Cre-loxP* technology to create a conditional *Nf1* allele (3). Importantly, the Parada's lab showed that the $Nf1^{flox}$ allele functions as a wild-type (WT) allele in spite of harboring *loxP* sites and a *neo* gene within its intronic sequences. The $Nf1^{flox}$ allele is readily recombined *in vivo* to make a null allele through co-expression of *Cre* recombinase. Drs. McClatchey, Jacks, and Giovannini used gene targeting to disrupt the *Nf2* locus (4, 5). Homozygous *Nf2* mutant embryos failed without initiating gastrulation. Although heterozygous *Nf2* mutant mice are cancer prone, these animals do not develop schwannoma or meningioma. To circumvent the early embryonic-lethal phenotype

associated with homozygous inactivation of *Nf2* and to test the hypothesis that the tumor spectrum might be modulated by the rate of the loss of the normal allele in specific tissues, Dr. Giovannini and his colleagues generated a conditional mutant *Nf2* allele (6). As expected, mice homozygous for the $Nf2^{flox2}$ mutant allele ($Nf2^{flox2/flox2}$) were viable and fertile suggesting that the introduction of *loxP* sites did not hamper *Nf2* expression. Induced expression of *Cre* recombinase in $Nf2^{flox2/flox2}$ mice results in biallelic inactivation of *Nf2* in specific tissues. A long standing research goal of this Consortium has involved exploiting these conditional mutant alleles of *Nf1* and *Nf2* to develop tractable models of NF-associated tumors for use in our labs and by other investigators. We make all strains that we develop available to academic investigators as soon as they are published with no restrictions. A list of investigators who have received these mice appears in the Reportable Outcomes section.

Models of NF1 and NF2-Associated Tumors. In work published prior to date, the participants in this consortium reported the phenotypic and biologic features of NF1-associated mouse tumor models of MPNST/Triton tumor, astrocytoma, JMML, plexiform neurofibroma, and chemotherapy-induced leukemia, sarcoma, and breast cancer (7-13) and of NF2-associated tumors such as vestibular Schwannoma and meningioma (5, 6). These data are also described in detail in previous Progress Reports.

Progress Report

Technical Objective (Aim) 1: To produce and characterize models of NF-associated tumors.

Signal Transduction in Primary *Nf1*-Deficient Hematopoietic Cells. In studies supported by a previous award to this consortium, the Shannon and Parada labs collaboratored to generate mice that develop a fatal myeloproleferative disorder (MPD) that models JMML by using the *Mx1-Cre* transgene to ablate *Nf1* in hematopoietic cells (14). This MPD is characterized by a subacute course with progressive leukocytosis and splenomegaly. *Mx1-Cre, Nf1*$^{flox/flox}$ mice succumb from this disease beginning around 6 months of age and ~80% are dead by one year. Bone marrow cells demonstrate hypersensitivity to the granulocyte-macrophage colony stimulating factor (GM-CSF) and form abnormally large myeloid progenitor colonies in methylcellulose cultures stimulated with saturating concentrations of myeloid growth factors (14). However, biochemical investigation of bone marrow cells from *Mx1-Cre, Nf1*$^{flox/flox}$ mice with MPD surprisingly revealed minimally elevated levels of the activated (phosphorylated) forms of the major Ras effectors MEK, ERK, and Akt. There are a number of potential explanations for these data including: (1) the experimental conditions used to starve and stimulate cells could have masked aberrant activation of effector cascades; (2) it is possible that primary *Nf1*-deficient cells respond to hyperactive Ras by remodeling signaling networks over time; and/or (3) hyperactive signaling within a minor subpopulation of immature myeloid cells might not be detected in lysates of whole bone marrow or spleen. The Shannon lab has thoroughly investigated these possibilities over the past two years, which has included a focused effort to adapt fluorescence activated cytometric (FACS) methodologies to analyze phosphorylated signaling molecules in subpopulations of hematopoietic stem and progenitor cells. The initial work focused on *Mx1-Cre, LSL-Kras*^{G12D} mice, which develop a more aggressive MPD than *Mx1-Cre, Nf1*$^{flox/flox}$ animals (15). A paper describing the FACS methodology and the effects of oncogenic Kras expression on signaling in hematopoietic stem/progenitor cells appeared earlier this year (16).

Developing Biochemical Signatures of *Nf1* Mutant Bone Marrow Cells. The Shannon lab first assayed signaling in primary WT and *Mx1-Cre, Nf1*$^{flox/flox}$ bone marrow (BM) cells under a variety of conditions and found that incubating BM cells in IMDM containing 1% BSA for 2.5 hours before stimulation gave consistent results without affecting cell appearance or survival (data not shown). To obtain a broad view of how *Nf1* inactivation modulates the signaling network in response to GM-CSF, the lab interrogated multiple signaling proteins commonly implicated in growth control. These studies were described in the 2006 Progress Report for this award. Under optimized conditions bone marrow cells from *Mx1-Cre, Nf1*$^{flox/flox}$ mice were starved in IMDM/BSA and stimulated with GM-CSF, 10% serum, or both to assess their specific contributions. Interestingly, cells from *Mx1-Cre, Nf1*$^{flox/flox}$ mice with MPD cells did not display increased basal levels of any signaling protein. Moreover, whereas some *Nf1* mutant BM samples showed normal or modestly elevated levels of phosphorylated signaling proteins (data not shown), there was striking attenuation in other experiments **(Fig. 1; next page).** This attenuated signaling phenotype was a consistent finding in mice with MPD on a uniform F1 strain background.

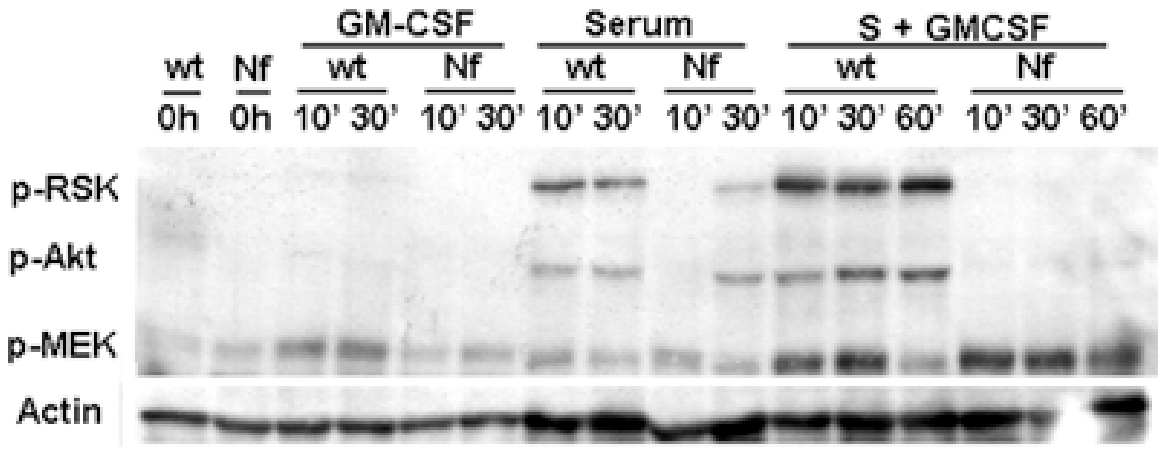


Figure 1. ***Attenuated Signaling in Nf1$^{-/-}$ mice.*** *Nf1$^{-/-}$ bone marrow cells that show dramatic attenuation in RSK and Akt phosphorylation.*

Multiparameter FACS Analysis of Hematopoietic Stem/Progenitor Cells *Mx1-Cre, Nf1$^{flox/flox}$* mice Mice with MPD. These provocative and unexpected results of raised the possibility data that *Nf1* mutant hematopoietic cells might adapt to hyperactive Ras signaling by dynamically remodeling signaling networks *in vivo*. Alternatively, the Western blot results might mask differences between immature stem/progenitor cells that are critical for disease and more differentiated cells. Unfortunately, Western blotting is impractical for interrogating the activation status of signaling molecules in small subpopulations of stem/progenitor cells because these cells are rare (1-2% of total bone marrow). FACS-based methodologies for measuring phosphorylated signaling proteins in defined subsets of hematopoietic cells overcome this limitation. The experimental protocol developed by the Shannon lab that is available through http://itsa.ucsf.edu/~kmslab/resources has now been utilized by many investigators. The current method for fixing and permeabilizing cells reliably detects the lineage-specific surface markers Mac1, Gr1, CD3, CD4, CD8, B220, and TER119, as well as the stem and progenitor cell marker c-kit. Cells defined by expression of c-kit and no (or low) expression of mature lineage markers (c-kit^{+} lin$^{-/dim}$) comprise 1-2% of nucleated marrow cells and are enriched for hematopoietic stem and progenitor cells. Although still diverse, this population is accessible for phospho-specific FACS analysis and allows comparison of signaling in WT and *Kras^{G12D}* cells with similar immunophenotypic and functional properties. The Shannon lab found that 3-10% of c-kit^{+} lin$^{-/dim}$ cells formed myeloid progenitor colonies in methylcellulose, and that the c-kit^{+} lin$^{-/dim}$ population included 85-94% of these cells within the marrow (16).

To further pursue the unexpected results shown in **Figure 1**, the Shannon lab compared phospho-protein levels in mature myeloid (Gr1^{+}/Mac1^{+}) and c-kit^{+} lin$^{-/dim}$ cells. As expected from the Western blotting results, unfractioned bone marrow and the mature myeloid population displayed lower levels of pERK and in response to GM-CSF (**Figs. 2; left and central middle panels**). Remarkably, however, a large proportion of *Nf1* mutant c-kit^{+} lin$^{-/dim}$ cells were hyper-responsive to GM-CSF (**Fig. 2; right middle panel**). Analysis of pS6 revealed a somewhat different pattern with increased levels of pS6 in whole bone marrow **(Fig 2; left lower panel)**, attenuated signaling in the mature marrow cells **(Fig 2; right lower panel)**, and hyperactivation in the c-kit^{+} lin$^{-/dim}$ stem/progenitor population c-kit^{+} lin$^{-/dim}$ cells. It is also interesting that *Nf1* inactivation and *Kras* oncogene expression did not have identical effects on cellular signaling networks. Whereas unstimulated c-kit^{+} lin$^{-/dim}$ cells from *Kras* mutant mice show elevated levels of phosphorylated ERK (16), this is not true of *Nf1*-deficient cells. Furthermore, downstream Ras

effectors are phosphorylated normally in *Kras* mutant BM that are stimulated with cytokines, while these responses are blunted in *Nf1* mutant cells. Interestingly, however, cells within the stem/progenitor compartment that play a central role in disease pathogenesis demonstrate aberrant Ras pathway hyper-activation in both *Nf1* and *Kras* mutant mice. The heterogeneous responses in discrete subsets of mutant cells have important implications for identifying therapeutic targets and monitoring responses to these agents.

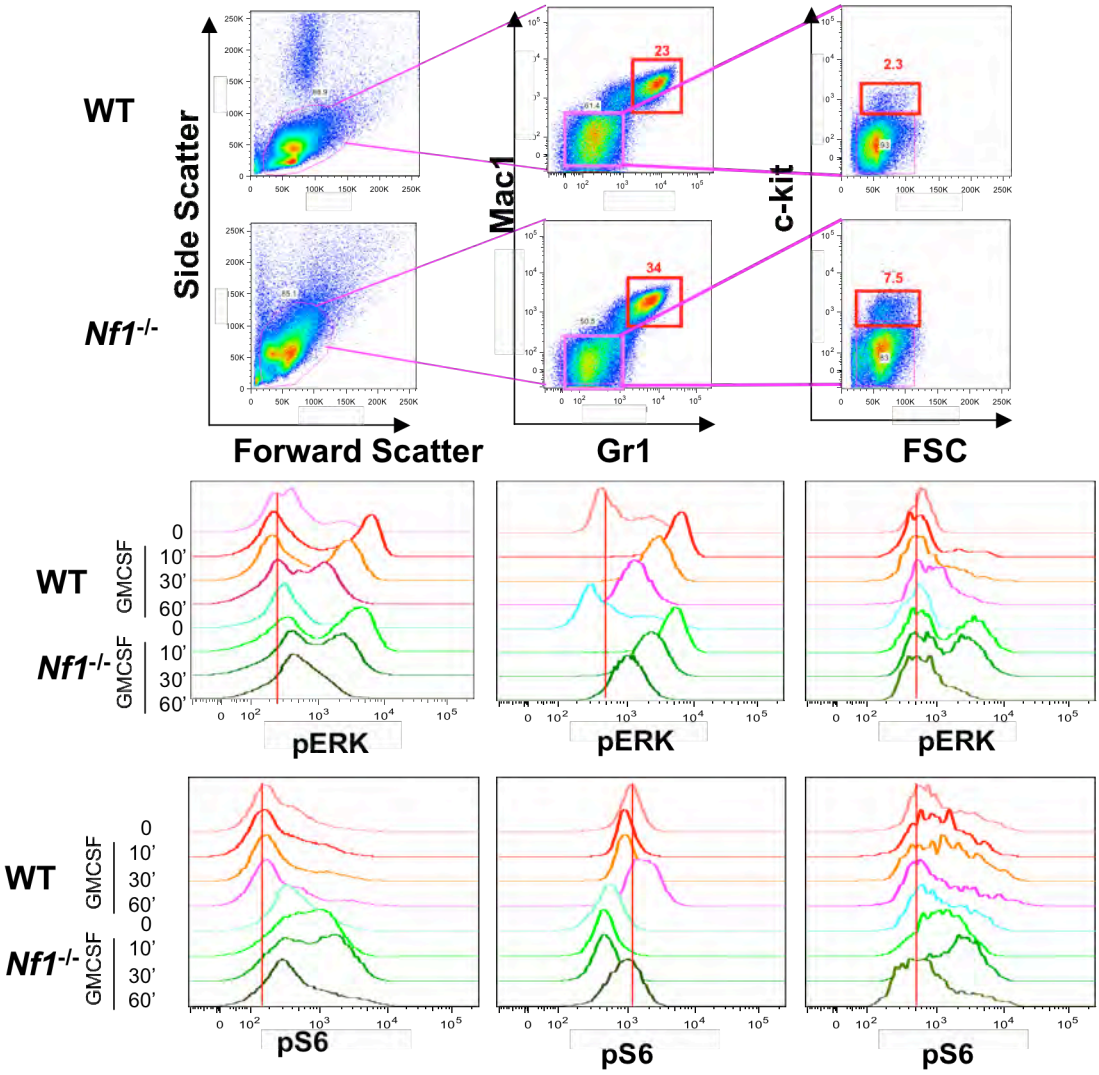


Figure 2. ***Signaling in Subsets of Nf1$^{-/-}$ Hematopoietic Cells***. *Top Panel. Gating strategy for interrogating whole marrow (left), mature myeloid cells (center), and stem/progenitor fractions. Middle Panel. Analysis of basal pERK levels and of changes over time after GM-CSF stimulation. Bottom Panel. Analysis of basal pS6 levels and of changes over time after stimulation.*

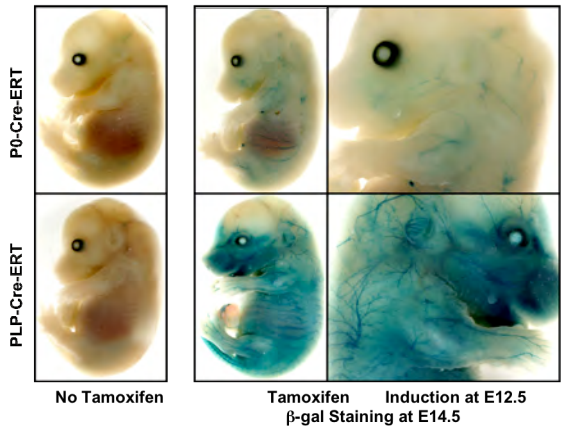
Local and Temporal Control of *Nf1* Inactivation. A major objective of the Parada lab has been to improve and refine existing mouse models of NF1. In previous work, the lab identified methods to develop plexiform neurofibromas as well as MPNSTs. However, in each instance, the time to tumor appearance was lengthy and variable. In addition, the use of the *Krox20-Cre* driver to

ablate *Nf1* rendered the mice somewhat sickly because of expression of these transgene in tissues irrelevant to current interests, but which could cause confounding side effects. The Parada lab has therefore worked extensively to improve the existing models.

The *Krox20-Cre* transgene has been valuable for ablating *Nf1* in SC and generating plexiform neurofibroimas and, subsequently, *Nf1; p53* mutant MPNSTs (8). The Parada lab uncovered interactions between tumorigenic cells and their surrounding microenvironment that are critical for tumor progression. Neurofibromas are complex multicellular tumors that include Schwann cells (SCs), fibroblasts, endothelial cells, and mast cells in the vicinity of peripheral nerves. Plexiform neurofibromas are histologically benign but can progress to malignancy. The Parada and Jacks labs showed that nullizygous loss of *Nf1* is required in the tumorigenic cell of origin SCs (Science papers). However, loss of heterozygosity in SCs is not sufficient for neurofibroma formation, and the Parada lab found that *Nf1* haploinsufficiency in at least one additional non-neoplastic lineage is required for tumor progression. Using adoptive transfer of hematopoietic cells, the lab established that haploinsufficiency of bone marrow derived cells in the tumor microenvironment is sufficient to allow neurofibroma progression in the context of SC nullizygosity (data not shown). Further, genetic or pharmacologic attenuation of the c-kit signaling pathway in hematopoietic cells greatly diminishes neurofibroma initiation and progression in *Nf1* mutant mice. These studies identify haploinsufficient hematopoietic cells and the c-kit receptor as therapeutic targets forinterfering with the growth of plexiform neurofibromas and implicate mast cells as critical mediators of tumor initiation. A manuscript describing this has been recently completed and submitted for publication with Dr. Parada and his collaborator Dr. Wade Clapp (Indiana University) as co-corresponding authors. A direct outcome of these murine experiments is an ongoing phase II clinical trial to treat plexiform neurofibromas at 2 centers. More that 30 patients are enrolled and preliminary observations support activity in some patients, including isolated cases of apparent tumor disappearance.

Drs. Giovannini and Parada are collaborating on studies in the *P0*-Cre strain generated by the Giovannini lab that shows a pattern of Cre expression restricted to SC precursors. These mice and others currently in use, permit *Nf1* inactivation at different stages of Schwann cell development. In all cases, *Nf1* loss at the progenitor stage leads to abnormal development of Remak bundles, the non-myelinated axon-SC unit, whose degeneration initiates neurofibroma formation. The progression to full-blown neurofibromas involves further degeneration of myelinating SCs and axons, which is dependent on the haploinsufficient tumor microenvironment. Thus, consistent with *Krox20-Cre* ablation of *Nf1* and with the genetic of human NF1, these additional studies confirm the requirement for heterozygosity of *Nf1* in neurofibroma formation.

A key refinement of the models developed by the Parada lab is the use of inducible recombination to refine the timing of tumor susceptibility and to identify the subpopulations of cells that have capacity to form tumors. For this the Parada lab introduced *P0* and *PLP*-Cre transgenic lines that are tamoxifen inducible **(Fig. 3; next page).** The relative inducibility *P0* (weak) and *PLP* (strong) facilitates testing this system in a variety of important ways. Again as described for the *Krox20* and *P0*-cre driven recombination, *PLP*-cre induced peripheral hyperplasia and plexiform tumor formation only in the context of a heterozygous environment **(Fig. 4; next page).** This refined model will permit more time-controlled preclinical studies.



. ***Figure 3***. ***P0 and PLP Cre-ERT transgenic embryos that also have a Rosa26 reporter for Cre activity.***

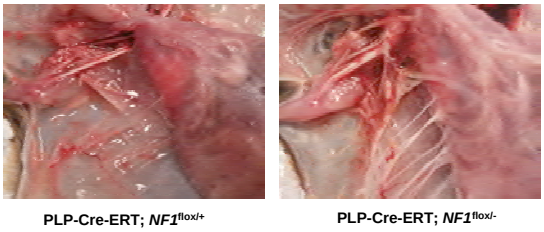


Figure 4. Nf1 Ablation at Immature SC Stage Results in Peripheral Nerve Hyperplasia. *Example of tamoxifen- induced nerve hyperplasia in PLP-Cre-ERT mice. These mice also develop plexiform neurofibromas.*

In recent studies, the Parada Lab has used a tamoxifen inducible CMV-Cre strain in which Cre recombinase is ubiquitously expressed to attempt to model dermal neurofibroma *in vivo* models. Topical application of tamoxifen on $Nf1^{flox/-}$ but not $Nf1^{flox/flox}$ mice induced neurofibromas that are indistinguishable histologically from their human counterparts **(Fig. 5).** The Parada lab will further evaluate this new and important model as incorporate them into preclinical studies over the next year.

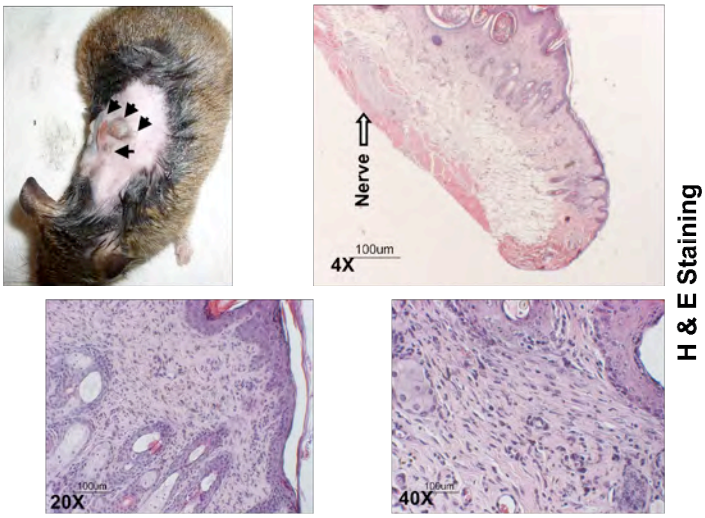


Figure 5. In vivo ablation of Nf1 in the skin induces dermal neurofibromas. *Experimental induction of dermal neurofibromas by topical application of tamoxifen. Histological stain shows association with peripheral nerve twig and classic features of neurofibromas.*

Use of Drug-Inducible Cre Mice to Locally and Temporally Control Deletion of the *Nf1* and *Nf2* Genes. The Jacks lab has generated tamoxifen-inducible Cre mice (Rosa26-$CreER^{T2}$) that permit local ablation at desired time points. These Rosa26-$CreER^{T2}$ mice were crossed to $Nf2^{flox}$ mice to generate Rosa26-$CreER^{T2}$; $Nf2^{flox/flox}$ mice. A single intraperitoneal injection of 3 mg/40 g body weight of tamoxifen into pregnant female mice at E8.5 resulted in neural tube defects in the subset of embryos with the genotype Rosa26-$CreER^{T2}$; $Nf2^{flox/flox}$. Thus, acute inactivation of *Nf2* in development can recapitulate the tissue fusion defects observed in Nestin-Cre^{p}; $Nf2^{flox/1lox}$

mice. The Jacks lab has also injected adult Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice with 1-3 intraperitoneal tamoxifen doses of 4.5 mg/40 g body weight. Mice receiving one intraperitoneal injection appeared healthy at 3 months; however, their coat color changed from solid black to patches of black and red. Mice receiving 3 injections of 4.5 mg/40g and aged for 6 months or more had more striking changes in fur coloration, a ruffled fur appearance, dermatitis, whisker loss, and multi-focal cholangiocarcinoma formation affecting the majority of bile ducts in the liver (**Fig. 6A**). Nervous system tumors were not detected. The Rosa26-CreER^{T2} mice will be used for several planned studies by the Jacks lab to investigate the requirement for the *Nf1* and *Nf2* tumor suppressors in adult tissues and to develop novel tumor models utilizing the ability to control timing and region of *Nf1* or *Nf2* loss (see below).

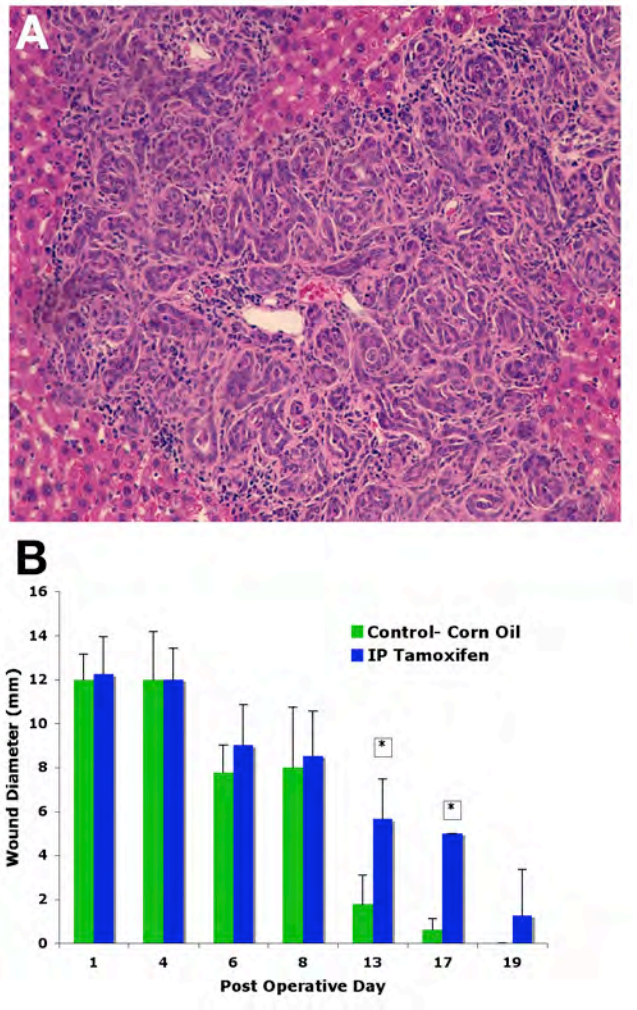


***Figure 6. Nf2 loss during adulthood leads to tumor formation and delayed wound healing. A**. Rosa26-CreER^{T2}; Nf2 $^{flox/flox}$ mice treated with 3 doses of tamoxifen (every other day, 4.5 mg per 40 g mouse) developed multifocal cholangiocarcinomas within 6 months following treatment. **B**. These mice demonstrated significantly delayed wound healing compared to control, vehicle-treated mice in a standard wound healing assay. A 1 cm square full thickness lesion was created in the dorsal skin and allowed to heal by secondary intention. While all wounds in control animals were healed by 3 weeks post-injury, some of the wounds in Rosa26-CreER^{T2}; Nf2 $^{flox/flox}$ mice remained unclosed even 2 months following injury.*

Generation and Characterization of Mouse Models of Ependymoma. Brain tumors are the second most frequent malignancy of childhood and are the leading cause of death from childhood cancer. Ependymomas are particularly common in young children, accounting for 6-12% of pediatric brain tumors, and this tumor type occurs at increased frequency in children with NF2. Although ependymomas are slow growing and histologically classified as WHO grade II/IV, the 5-year progression free survival is only 50%, with children under two years of age having a particularly dismal prognosis.

Since mutation of the *NF2* gene is the only well documented genetic alteration in human ependymomas, the Jacks lab is generating mouse models of ependymoma in which the initiating event is loss of *Nf2*. Previous work from the Jacks lab showed that $Nf2^{-/-}$ mice die in early embryogenesis due to defects in the extraembryonic tissues, and that $Nf2^{+/-}$ mice develop malignant tumors but not the tumor types characteristic of the human disease (schwannoma, meningioma, and ependymoma) (4, 17). Therefore, the Jacks lab used the conditional $Nf2^{flox}$ allele generated by Dr. Giovannini, and the transgenic Nestin-Cre and *h*GFAP-Cre mice in which Cre is expressed within ependymal cells but also within other cells of the body and/or brain. Nestin-Cre expression is detected in the neuroepithelium as early as E8.5, and is expressed in a mosaic pattern throughout the body of the embryo. *h*GFAP-Cre expression is restricted to neurons and glial cells, and is detected as early as E14.5. The Jacks lab previously showed that Nestin-Cre; $Nf2^{flox/flox}$ mice in which the neural tube has closed (70% of all mutants) showed diffuse hyperproliferation of ependymal cells around the ventricles and central canal of the spinal cord. A single Nestin-Cre; $Nf2^{flox/flox}$ mouse that survived 11 days past birth had a focal lesion arising from the wall of the third ventricle in which the ependymal cells formed tubules resembling the true rosettes of human ependymomas. This lesion appears to represent the earliest stage of ependymoma. By contrast, the Jacks lab has generated 25 *h*GFAP-Cre; $Nf2^{flox/flox}$ mice, and to date none of the mice analyzed histologically have shown evidence of ependymal cell hyperproliferation. These studies suggest that loss of *Nf2* in neuroepithelial cells (precursors to ependymal cells) is a more efficient way to generate ependymomas, or that loss of *Nf2* prior to neural tube closure is required for tumor initiation. However, the early lethality of Nestin-Cre; $Nf2^{flox/flox}$ mice precludes its use as an ependymoma model.

Since *h*GFAP-Cre; $Nf2^{flox/flox}$ mice do not develop ependymoma, the Jacks lab has investigated whether mutations in other genes may cooperate with *Nf2* inactivation in tumor initiation. Toward this end, cohorts of 25 *h*GFAP-Cre; $Nf2^{flox/flox}$; $p53^{flox/flox}$ mice and 25 *h*GFAP-Cre; $Nf2^{flox/flox}$; $APC^{flox/flox}$ mice have been generated, as both *p53* and *APC* mutations are implicated in the pathogenesis of human ependymomas. None of these mice, which have been aged as long as 18 months, have developed ependymomas. Based on reports in the literature that loss of *Nf2* may synergize with oncogenic mutations in *Kras*, the Jacks lab also generated >25 *h*GFAP-Cre; $Nf2^{flox/flox}$; $Kras^{G12D}$ mice. $Kras^{G12D}$ mice conditionally express oncogenic K-Ras^{G12D} in the presence of Cre recombinase. The Jacks lab has seen evidence of ependymal hyperproliferation in these mice by 6 weeks of age, suggesting that *h*GFAP-Cre; $Nf2^{flox/flox}$; $Kras^{G12D}$ mice may yield a tractable model of ependymoma. Identifying these hyperplastic regions within the ependyma requires serial, 100 μm sections; therefore, analysis of the frequency of such lesions is ongoing. In order to follow the time course of ependymoma formation, animals were aged as long as possible. Approximately half of the mice with the *h*GFAP-Cre; $Nf2^{flox/flox}$; $Kras^{G12D}$ genotype developed severe, ulcerating dermatitis that required them to be sacrificed near 6 weeks of age. Others failed to develop dermatitis, but developed well-circumscribed subcutaneous tumors (sarcomas) at 4-6 months of age that quickly enlarged to greater than 1 cm in diameter, ulcerated through the skin and required the animal to be euthanized. The *h*GFAP-Cre; $Nf2^{flox/flox}$; $Kras^{G12D}$ mice were generated on a mixed 129/B6 background that most likely accounts for these phenotypic differences. All ependymal hyperplasia thus far identified was seen in the mice that developed severe dermatitis by 6 weeks. These mice also had an abnormal, syndromic appearance with runted size and shortened snout. In addition, these mice develop widespread tumor formation within cranial nerves. Together these results suggest that expressing K-Ras^{G12D} in the context of *Nf2* inactivation may be a novel

strategy for modeling the tumors seen in NF2 patients. The Jacks lab is currently generating Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice to study the role of interaction between these mutations in tumor formation in other tissues.

A Mouse Model to Study Adult Onset Tumors in NF1. Significant advances have been made by members of this consortium to model nervous system tumors including MPNST/Triton tumors and plexiform neurofibromas that typically arise congenitally in NF1 patients. How *Nf1* loss results in tumor formation in adulthood, however, has not been substantially addressed. This issue is of particular importance to patients given that subcutaneous neurofibromas, which typically arise during puberty, may result in significant disfigurement due to their presence in large numbers and discomfort from intense pruritis. These tumors also appear to differ fundamentally from congenital neurofibromas as they lack the ability to undergo malignant transformation. A mouse model of subcutaneous neurofibromas will be crucial for gaining a better understanding of factors leading to tumor initiation and potential mechanisms of prevention. In addition, the ability to contrast congenital with adult onset neurofibromas in a mouse model may yield insight into the mechanisms of malignant transformation. Of particular interest is whether developmental properties of the cell of origin may dictate the ultimate malignant potential of the tumor. Neurofibromas are thought to arise from SCs; however, it remains possible that congenital tumors may arise from SC precursors or stem cell population present only during early development.

The Jacks lab is currently generating a mouse model that will allow careful control of the timing of *Nf1* loss by crossing Rosa26-CreER^{T2} mice with conditional *Nf1* $^{flox/+}$ and *Nf1*$^{+/-}$ mice to generate Rosa26-CreER^{T2}; *Nf1*$^{flox/-}$ mice. These mice will effectively be heterozygous *Nf1* mutant, which has previously been shown by Dr. Parada to be required for formation of neurofibromas. *Nf1* will then be lost in sporadic cells following tamoxifen administration. Neurofibromas will be induced at different stages of development by administering tamoxifen either systemically or through the skin by dissolving in DMSO. Approximately 25 Rosa26-CreER^{T2}; *Nf1*$^{flox/-}$ mice have been generated, treated with tamoxifen at 6-8 weeks of age and are currently aging. After 4-6 months, mice have not developed any gross dermal neurofibromas. Additional mice have been generated which will be treated at earlier developmental time points including embryonic day 17 (by giving i.p. tamoxifen to pregnant dams) and postnatal days 3, 10, and 21 to determine whether *Nf1* loss from a cell type present earlier in development may be required for the tumors to then develop during adulthood. Dermal neurofibromas have been particularly difficult to model, raising the possibility that mice may be resistant to this type of tumor formation. However, Dr. Yuan Zhu at University of Michigan has recently developed a model that develops dermal neurofibromas with nearly a 100% penetrance. These mice have the genotype of P0(B)-Cre, *Nf1*$^{flox/-}$; utilizing the A strain of P0-Cre transgenic mice originally made by Dr. Giovannini's laboratory. Dr. Zhu has generously shared this strain with the Jacks laboratory, and it is currently being rederived in our mouse facility. Although the timing of *Nf1* inactivation (early development) and eventual tumor onset cannot be controlled in this mouse, it will be useful for comparing to the tumors that arise in our conditional system.

Technical Objective (Aim) 2: Consequences of Nf1 and Nf2 Inactivation and Therapeutic Target Identification

Effects of Inhibiting p120GAP on the Growth of *Nf1* Deficient Cells. Most mammalian cells express two major GTPase activating proteins – neurofibromin and p120GAP, which have distinct binding affinities and catalytic activities (18, 19). *Nf1* deficient cells only produce p120GAP and presumably rely upon this protein to control Ras activity. This raised the possibility that normal cells would tolerate transient inhibition of p120GAP because they retain neurofibromin, whereas this might induce catastrophic Ras activation in *Nf1*-deficient cells. The reverse is also possible – that *Nf1* mutant cells will proliferate more vigorously if p120GAP is ablated. The Shannon lab exploited siRNA technology to test these alternative models in cells from *Nf1* mutant mice. Three siRNA constructs that target different regions of the p120GAP mRNA were developed and cloned into the murine stem cell virus (MSCV) retroviral backbone. As described in the previous Progress Report for this award, mouse embryonic fibroblasts (MEFs) that were infected with these vectors showed variable reductions in p120GAP levels. Knocking down p120GAP expression also reduced proliferation in MEFs (**Fig. 7, left panel**), and β galactosidase staining suggested increased levels of senescence. Interestingly, the effect of p120GAP knockdown was not significantly different between WT and *Mx1-Cre* $Nf1^{flox/flox}$ MEFs. Consistent with these results, the Shannon lab also found that knocking down expression markedly reduced the growth of myeloid progenitor colonies from both WT and *Mx1-Cre* $Nf1^{flox/flox}$ hematopoietic cells over a range of GM-CSF concentrations (**Fig. 7, right panel).** The inhibitory effects of p120GAP siRNAs observed to date are observed in both WT and *Nf1* mutant cells. Together, the siRNA data suggests that p120GAP is both a negative regulator of Ras signaling and may play an essential role in transducing proliferative signals, perhaps by interacting with Ras-GTP. If this idea is true, it could explain why *NF1* functions as a tumor suppressor gene in humans, while *GAP* does not. These hypotheses can be tested by performing experiments that are beyond the scope of the current project such as assessing the effects of reducing p120GAP expression on hematopoietic reconstitution in a transduction/transplantation assay or by constructing mice with a conditional *Gap* mutation. Since p120GAP siRNAs did not show selective effects on the growth of *Nf1* mutant MEFs or hematopoietic progenitors, the data do not support the hypothesis that inhibitors of p120GAP might prove beneficial for treating the complications of NF1 disease.

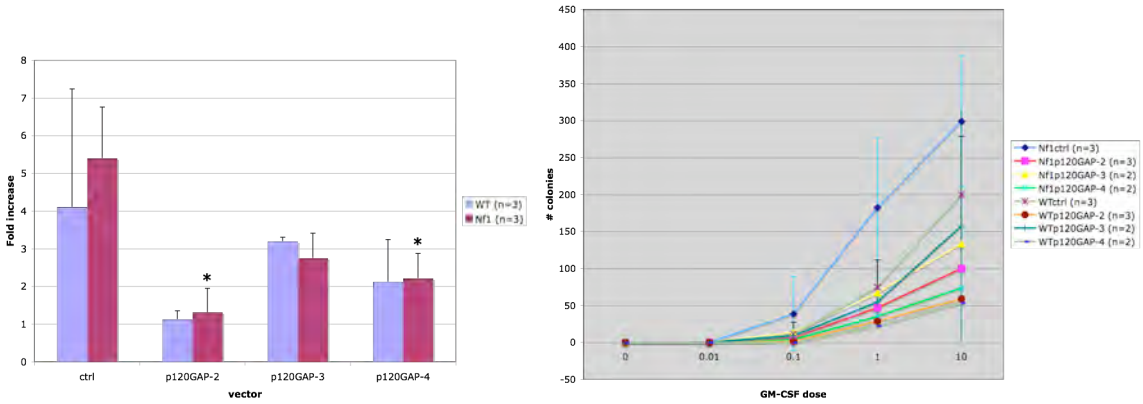


Figure 7*. Effects of p120GAP Knockdown on Cell Growth. A.** *Two independent shRNAs markedly reduced MEF growth.* ***B. *p120GAP knockdown reduces the growth of normal and Nf1 deficient hematopoietic progenitors.*

Treating *Nf1* Deficient and WT Hematopoietic Cells with Inhibitors of Ras Effectors. Since Ras-GTP activates multiple downstream signaling pathways, a central question in developing effective NF1 treatments involves determining which of these cascades mediates aberrant growth *in vivo.* Furthermore, effective agents must show a beneficial therapeutic by selectively inhibiting the growth of diseased cells without damaging normal tissues. The Shannon lab is making a major effort to utilize strains of mice developed by this consortium to address these general questions in models of MPD and acute myeloid leukemia (AML). The hematopoietic system offers distinct advantages for drug screening efforts such as: (1) disease status can be monitored easily by visual inspection of mice and by performing a simple complete blood count; (2) leukemic cells can be transplanted into irradiated recipient mice to create matched control animals for studies comparing treated versus untreated groups; (3) robust *in vitro* assays exist for comparing the growth of normal and diseased cells; (4) methods are well established for purifying stem and progenitors cells at discrete stages of differentiation, including cancer stem cells; (5) it is feasible to monitor the activation of target proteins and pathways in primary cells and to directly assess the biochemical effects of specific agents; (6) it is straightforward to introduce genes into hematopoietic cells; and, (7) powerful methodologies exist for performing forward genetic screens to identify candidate genes that cooperate with *Nf1* loss in tumorigenesis and to uncover mechanisms of resistance.

Retroviral insertional mutagenesis (RIM) is a powerful *in vivo* strategy for performing forward genetic screens to identify mammalian cancer genes. Retroviral replication requires the stable integration of a DNA provirus into the host genome. Retroviruses induce cancer when proviral sequences act as insertional mutagens that deregulate proto-oncogene expression or inactivate tumor suppressor genes. By providing a molecular sequence tag where it inserts, the provirus facilitates isolating genes that contribute to malignant growth. Pioneering studies performed in the laboratory of Neal Copeland and Nancy Jenkins that identified oncogenes and tumor suppressors such as *Myc*, *Evi1, Hoxa9* and *Nf1* have illustrated the power of this approach (20). Widespread use of this method was originally impeded by inefficient cloning techniques and lack of mouse genome sequence. These bottlenecks were overcome by the completion of mouse genome and PCR-based advances in cloning retroviral-genome DNA integrations. As technologies for cloning proviral insertion sites and mapping relevant target genes advanced, the strains used to perform RIM screens emerged as a rate-limiting step. To overcome this obstacle, Lynda Wolff created a replication competent ecotropic MuLV called MOL4070LTR (21). The recombinant virus is NB-tropic; however, unlike Moloney MuLV, MOL4070LTR efficiently induces AML with diverse cellular phenotypes in WT FVB/n and 129/Sv mice. Moreover, heterozygous $p15^{Ink4b}$ mutant mice that were infected with MOL4070LTR immediately after birth showed increased susceptibility to myeloid leukemia compared to WT littermates (21, 22). Similarly, the novel candidate oncogenes *Plag1* and *Plag2* were identified in knock in mice engineered to express Cbfb-MYH11, a human myeloid leukemia fusion protein associated the inv(16)(p13q22), that were infected with virus 4070A (23). These data both confirm the wide applicability of MOL4070LTR as a tool for efficiently performing RIM screens in the context of oncogenes and tumor suppressors and underscore the potential value of this model to identify novel genetic pathways that contribute to leukemogenesis.

The Shannon is performing RIM with MOL4070LTR in *Mx1-Cre*, $Nf1^{flox/flox}$ mice to uncover mutations that cooperate with *Nf1* inactivation in progression from MPD to AML. The lab first injected 46 *Mx1-Cre*, $Nf1^{flox/flox}$ and 48 control $Nf1^{flox/flox}$ littermates with both MOL4070LTR and pI-pC at day 3 to 5 of life. The unrearranged $Nf1^{flox}$ allele, which produces

normal levels of neurofibromin, remains intact in *Nf1*$^{flox/flox}$ mice that do not inherit the *Mx1-Cre* transgene. *Mx1-Cre, Nf1*$^{flox/flox}$ mice demonstrated a higher incidence of acute leukemia and reduced latency (**Fig. 8A**). Overall, 9 of 46 *Mx1-Cre, Nf1*$^{flox/flox}$ mice developed AML versus 4 of 48 cases of AML in the control group, with myeloblasts visible in blood and marrow and myeloid markers detected by FACS (**Figs. 8B, 8C**). Whereas the MPD is only transplantable into lethally irradiated hosts, the sublethally irradiated recipient mice injected with AML cells develop disease with the same immunophenotype as the original leukemias within 3-6 weeks (**Fig. 8D**). The Shannon lab recently generated *Mx1-Cre* transgenic mice with one conditional and one germline mutant *Nf1* allele on a C57Bl/6 x 129Sv/J F1 strain background. Injecting these mice with MOL4070LTR has markedly increased the efficiency of the RIM procedure with ~50% of mice developing AML. To date, the Shannon lab has cryopreserved ~20 additional AMLs from *Mx1-Cre, Nf1*$^{flox/flox}$ and *Mx1-Cre, Nf1*$^{flox/-}$ mice as well as AMLs from control mice that were injected with MOL4070LTR. These transplantable cancers are an exceptional resource for preclinical studies of response and resistance to targeted agents.

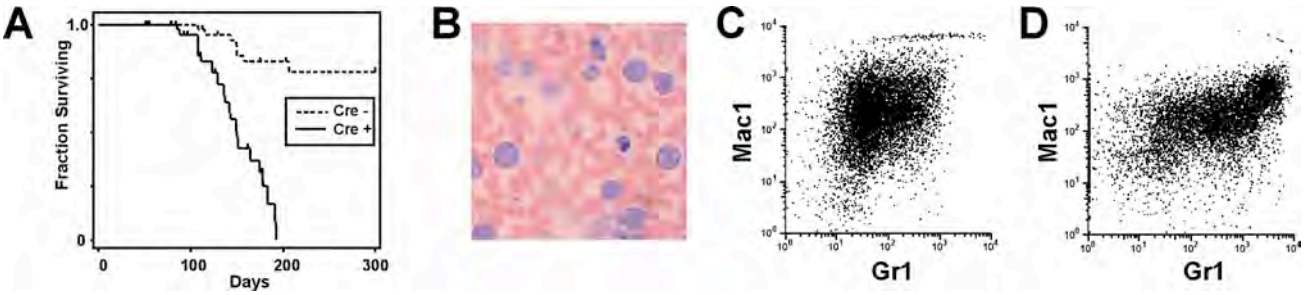


Figure 8. ***AML in Mx1-Cre, Nf1***$^{flox/flox}$ ***Mice***. **A.** *Survival of Mx1-Cre*$^{+}$ *and control Cre*$^{-}$ *mice*. **B.** *Blood smear showing myeloblasts.* **C and D.** *Flow cytometry demonstrating Mac1+/Gr1*^{lo} *phenotype of myeloblasts in a primary AML (C) and a transplant recipient (D).*

Preclinical Evaluation of a MEK Inhibitor in *Mx1-Cre, Nf1*$^{flox/flox}$ Mice with MPD and AML. MEK is a key component of the Ras/MEK/ERK cascade, which is a major effector pathway downstream of Ras-GTP. The Shannon lab obtained the MEK inhibitor CI-1040 from Pfizer, Inc., and found that 25-50 μM of CI-1040 abrogated CFU-GM colony formation from *Mx1-Cre, Nf1*$^{flox/flox}$ bone marrow in response to GM-CSF. Importantly, however, there was no therapeutic index as CFU-GM growth from WT bone marrow was inhibited at similar concentrations (**Fig. 9A**). In a randomized preclinical trial, *Mx1-Cre, Nf1*$^{flox/flox}$ mice with MPD that received CI-1040 at the MTD (100 mg/kg twice a day) showed no improvement in leukocyte counts or splenomegaly. Pharmacodynamic analysis demonstrated transient inhibition of MEK kinase activity in bone marrow cells from CI-1040-treated mice, which was assessed by measuring the ability of GM-CSF to increase the levels of phosphorylated ERK (pERK). Whereas pERK levels were markedly decreased 2 hours following a CI-1040 dose, inhibition was estimated to be ~50% after 4 hours and was no longer detected after 8 hours (data not shown).

The Shannon lab next evaluated *in vitro* CI-1040 responses of AMLs that were generated by injecting *Mx1-Cre Nf1* $^{flox/flox}$ mice with the MOL4070LTR retrovirus. Unexpectedly, colony growth was completely abrogated at much lower drug concentrations (0.25- 5 μM) than in WT or MPD bone marrows, suggesting that *Mx1-Cre Nf1* $^{flox/flox}$ AMLs are more sensitive to MEK inhibition (**Fig. 9A**). To test this hypothesis directly, the Shannon lab performed a preclinical trial in 25 recipient mice that were transplanted with 4 independent leukemias. These mice were

randomized to treatment with either CI-1040 or control vehicle when flow cytometry revealed blast cells in the peripheral blood, which occurred 2-4 weeks after transplantation. CI-1040 had dramatic effects in this setting. Whereas leukocyte counts increased progressively in the vehicle-treated mice, CI-1040 treatment consistently induced a reduction in circulating leukocytes (**Fig. 9B**) and was associated with markedly prolonged survival (24 versus 7 days in the vehicle-treated cohort; odds ratio 3.5, 95% CI 3.0-3.8; see **Fig. 9C**). These data demonstrate that the biologic response to a molecularly-targeted inhibitor is strongly modulated by the genetic context in which a disease-initiating mutation occurs. Specifically, although the MPD induced by *Nf1* inactivation does not respond to CI-1040, progression to AML is associated with enhanced sensitivity to this agent.

Mice with AML that receive CI-1040 consistently show a decrease in blood leukocyte counts with reappearance of mature myeloid cells, improved physical condition, and prolonged survival. However, these animals eventually develop leukocytosis with reemergence of peripheral blood blasts, and die with AML. These leukemias are remarkably less sensitive to CI-1040 *in vitro* than the parental AMLs (**Fig. 9D**), and do not respond to treatment in secondary recipients. Interestingly, we observe equivalent inhibition of pERK in sensitive and

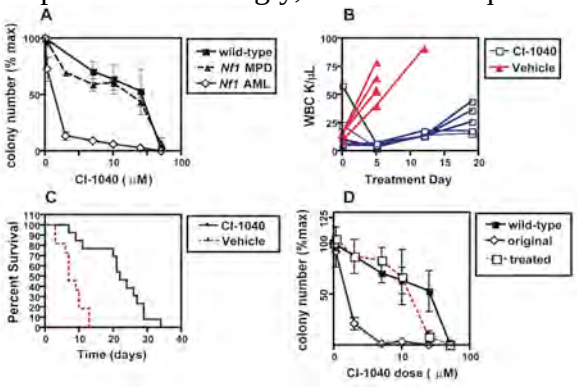


Figure 9. Preclinical evaluation of CI-1040 in murine AML. **A.** *Myeloid colony formation in methylcellulose from Mx1-Cre* $Nf1^{flox/flox}$ *mice with MPD or AML and WT bone marrow over a range of CI-1040 concentrations.* **B and C**. *White blood cell (WBC) counts (B) and survival (C) in recipient mice with AML that received CI-1040 or control vehicle*. **D.** *Relapsed AMLs show robust CFU-GM growth compared to original leukemias in presence of CI-1040.*

resistant AMLs that are exposed to a range of CI-1040 concentrations (data not shown). These latter data infer that resistance is not due to acquired *MEK1* mutations that render the kinase insensitive to the drug. Importantly, CI-1040-resistant AMLs contain new retroviral integrations (**Fig. 10**). Together, these data strongly support the hypothesis that these leukemias undergo clonal selection *in vivo* during treatment. To identify genes that might modulate resistance to CI-1040, a "shot gun" strategy was adapted to clone retroviral integrations. This methodology has the advantage of identifying the overall frequency of each integration. The Shannon lab then compared integrations from independent pairs of parental (sensitive) and resistant leukemias. These studies have uncovered strong candidate resistance genes that include members of the *RasGRP* family and *Mapk14*, which encodes p38.

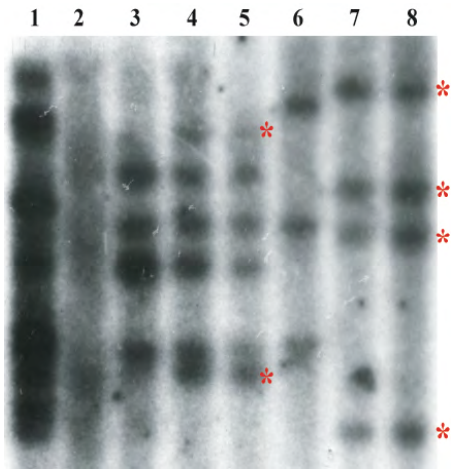


Figure 10. Integration patterns of two sensitive Nf1 AMLs (#6554 in lane 3; #6537 in lane 6) and resistant AMLs recovered from recipients that were treated with CI-1040 (#6554 in lanes 4 and 5; #6537 lanes 7 and 8). *MOL4070LTR viral producer cell line (lane 1) shows stable clonal integrations while WT bone marrow (lane 2) shows no endogenous integration sites. Novel integrations (*) are present in CI-1040 resistant leukemias and shared between resistant variants isolated from independent mice, which indicates that these resistant subclones are present at low levels in the primary sensitive AML.*

Together, these studies of CI-1040: (1) suggest that *in vitro* screens based on the ability of a compound to selectively inhibit the growth of primary *Nf1* mutant progenitors may predict *in vivo* efficacy; (2) affirm the importance of monitoring biochemical targets in tumor cells; (3) establish a tractable system for isolating genes that modulate both the response to a targeted agent and resistance; and, (4) show that stage of disease (early versus advanced) can influence the efficacy of a targeted agent. This latter observation may prove to be highly relevant to evaluating new treatments for other NF1-associated tumors where an early stage lesion such as plexiform neurofibroma can progress to MPNST. In recent studies, the Shannon has investigated PD325901, a newer compound that demonstrates prolonged MEK inhibition when administered once per day. They defined 12.5 mg/day as the maximally tolerated dose (MTD) normal and leukemic mice, and found that doses of 5.0 – 12.5 mg/kg/day induced regression of primary *Nf1* mutant AMLs *in vivo* (data not shown). Based on these data, PD325901 is being used in ongoing preclinical studies as a single agent in combination with other agents in MPDs and AMLs characterized by *Nf1* inactivation.

Signaling Networks in *Nf1* Mutant AMLs. The Shannon lab is integrating biochemical and therapeutic response data from *Nf1* mutant mice. In a simple "additive" model of multistep tumorigenesis, *Nf1* inactivation is predicted to result in deregulated Ras signaling whereas cooperating genetic lesions confer other phenotypes such as impaired differentiation, invasiveness, or enhanced self-renewal. However, it is also possible that secondary mutations influence the effects of *Nf1* loss on signaling. Indeed, recent studies by the Shannon lab support this latter model as both Western blotting and phospho-signaling analysis of primary AMLs from *Nf1* mutant mice revealed dramatic modulation of Raf/MEK/ERK and PI3 kinase/Akt signaling in comparison to both WT and MPD marrows (**Fig. 11**). The Shannon lab will analyze additional leukemias and will correlate signaling aberrations with therapeutic responses and patterns of retroviral integrations in these cancers.

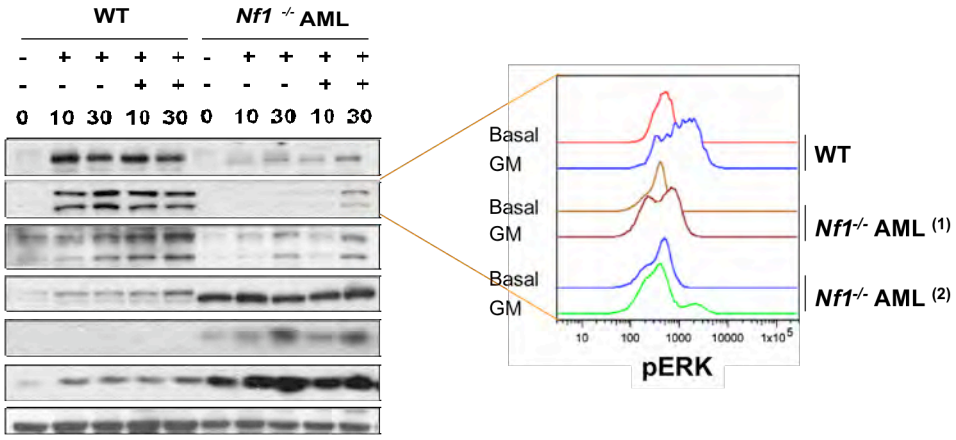


Figure 11. Signaling in Nf1-/- AMLs Induced by RIM. Note that the ability of GM-CSF to activate some, but not all, Ras effectors is markedly blunted. *pERK is highlighted here as analyzed by Western blotting (left) and flow cytomery (right).*

Combining Mouse Models, Functional Genomics, and Cell Lines to Identify and Target Potential Therapeutic Pathways in Neurofibroma. The Parada lab has developed a system for accurately

generating meaningful functional genomic data from NF1-associated tumors. Neurofibromas are complex tumors comprised of many cell types. The Parada lab identified the SC lineage as the critical target of *Nf1* inactivation in neurofibroma, and showed that the mast cell is a essential accomplice. To learn more about the important genes that induce the SC tumor phenotype, it is essential to compare them directly to the tumor cell of origin. The Parada lab recently succeeded in identifying the precursor populations that give rise to neurofibromas. The experimental approach involves isolating cells from *Nf1*$^{flox/-}$ mice, inducing LOH with a variety of techniques in culture such as viral infection or tamoxifen administration, and reintroducing nullizygous progenitors back into the sciatic nerves of the same mouse from which we isolated the precursors. Unmanipulated control cells are introduced in the other sciatic nerve. In 100% of cases, the sciatic nerve receiving the precursor cells in which *Nf1* had been inactivated *ex vivo* develop plexiform neurofibromas whereas the unmanipulated precursors did not form tumors **(Fig. 12).** This exciting advance provides a controlled system for generating genomic and biochemical data comparing the precursor non-tumorigenic cells to the product tumor. This approach should greatly enhance our ability to uncover important pathways and therapeutic targets. Similar approaches but including a conditional *Tp53* mutant allele yields precursors that give rise to neurofibromas when implanted, which subsequently progress to MPNSTs. The Parada lab can now separate the benign from the malignant tissue macroscopically and will therefore has another precursor/product set of tissues to compare directly.

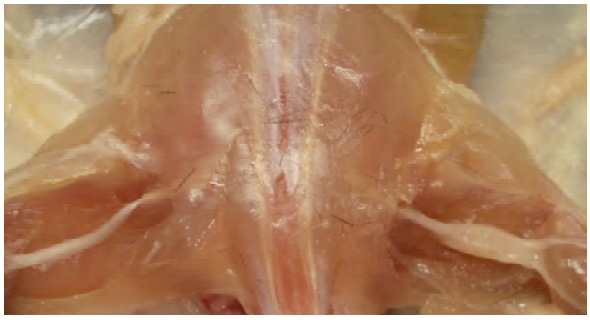


***Figure 12*. Reimplantation of neurofibroma precursor cells in left sciatic nerve and in vitro recombined precursors in right sciatic nerve of CMV-CreERT;NF1 $^{flox/-}$ mice.** *Only the right sciatic nerve gives rise to plexiform neurofibroma and this is dependent on LOH of NF1 and on a heterozygous environment.*

Characterizing the Role of *Nf2* in Wound Healing. The results of the Jacks laboratory demonstrating a role for merlin in regulating tissue fusion raises the possibility that it may also be important in normal wound healing, which occurs by similar mechanisms. Moreover, merlin is both a target and a regulator of Rac, a crucial protein for cell motility, migration, and proliferation that is also implicated in tumor progression. Prior work from the Jacks lab demonstrated that *Nf2*$^{-/-}$ MEFs had features consistent with hyperactivation of Rac, and displayed an enhanced migratory response in an *in vitro* wound healing assay resulting in wound closure 2-3 times faster than control MEFs. Together, these data suggest that wounds deficient in *Nf2* may close faster as a result of increased migration, but have increased apoptosis and poor strength due to inability to form appropriate cell-cell junctions. In addition, it was unclear whether wounded skin might be prone to tumor formation as epidermal cells unable to form normal adherens junctions might be prone to unchecked proliferation.

To test these hypotheses, a cohort of Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice were treated with tamoxifen (3 intraperitoneal doses of 4.5 mg per 40 g body weight) and tested in a standard wound healing assay by placing a 1 cm square wound on the back and allowing it to heal by

secondary intention. Wounds of experimental animals were compared to Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice that received vehicle alone or *Nf2*$^{flox/flox}$ mice given the same dose of tamoxifen. Wounds in Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice treated with tamoxifen were found to have significantly delayed healing as compared to control mice (**Fig. 6B above**). Some wounds remained unhealed after >2 months, others demonstrated a wider diameter but reduced thickness of scar tissue. Wounded mice were allowed to age for several months, but no tumors at the wound site have been observed. However, as they aged, Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice treated with systemic tamoxifen developed other significant systemic disease as described above including change in fur color and luster, severe dermatitis at areas of excoriation, whisker loss, and multi-focal cholangiocarcinoma formation affecting the majority of bile ducts in the liver and comprising more than half of total liver cells (**Fig. 6B**). As a result, it was not possible to determine whether the delayed wound healing was due to overall poor health of the animal or changes in epithelial cell function. The Jacks lab is currently evaluating several methods to specifically delete *Nf2* from the skin using topical application of 4-hydroxytamoxifen on the backskin of Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice. A cohort of Keratin-14- CreER^{T2}; *Nf2*$^{flox/flox}$ mice are also being generated using the Keratin-14- CreER^{T2} strain initially made in Elaine Fuchs laboratory. This promoter will allow for epithelial specific deletion within the skin. Ultimately, the results of these studies should yield insights not only into the mechanisms of wound healing, but also to the role of *Nf2* in metastasis.

Investigating a Potential Role of Injury in NF2 Tumor Initiation. The presence of bilateral vestibular Schwannomas is diagnostic for NF2, and mutations in *Nf2* are frequently seen in sporadic cases of these tumors. While tumors affecting the spinal roots, cranial nerves V, IX, or X occur, they are much less common, raising the possibility that cranial nerve VIII is particularly susceptible to tumor formation. Tumors are thought to arise from loss of the normal *Nf2* allele, but an additional inciting event may also be required. One hypothesis, given the tortuous path that cranial nerve VIII follows through the skull, is that injury may play a role in stimulating tumor formation. To test this hypothesis, the Jacks lab has generated a cohort of Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice, administered 4-hydroxytamoxifen to the injury site and allowed the animals to age to monitor tumor formation. Most studies have been performed in a sciatic nerve injury model to test the role of injury in schwannoma formation due to the accessibility of this site. A 3 mm segment of nerve was from the mid-thigh to prevent regrowth following injury. Cranial nerve injuries were similarly performed using the aid of a dissecting microscope, where the facial nerve (CNVII) was selected due to its accessibility. Given the evidence of a potential synergism between oncogenic *Kras*^{G12D} and *Nf2*-loss in generating ependymal hyperplasia and cranial nerve tumors (discussed above), Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice are being evaluated simultaneously in these injury studies. Sham-operated nerves administered 4-hydroxytamoxifen have been used as controls. While the majority of the cohort continues to age, early evidence suggests that injury may play a key role in tumor formation with tumors developing within the proximal nerve stumps within months in injured Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice and within weeks in Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice (**Fig. 13**). Unfortunately, spread of tamoxifen to non-nerve sites in the Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice results in papilloma formation, particularly on the mouth, requiring the animals to be euthanized 4-6 weeks following nerve injury and tamoxifen administration. As a result, the Jacks Lab is currently working to develop methods that will allow more localized delivery of tamoxifen and/or Cre-mediated excision through the use of viral vectors.

Finally, the Jacks Lab is testing a brain injury model whereas adeno-Cre is injected into the lateral ventricle to determine whether injury and disruption of cell-cell junctions in the ependyma is sufficient to induce ependymoma formation in the context of *Nf2*-loss. As the number of ependymal cells that undergo recombination may be limited using this method, *h*GFAP-Cre; *Nf2* $^{flox/flox}$ mice will also be tested in this injury model. The Jacks lab has previously found that complete recombination is achieved by birth using *h*GFAP-Cre crossed onto a reporter strain. Mice involved in these initial brain injury studies are currently aging.

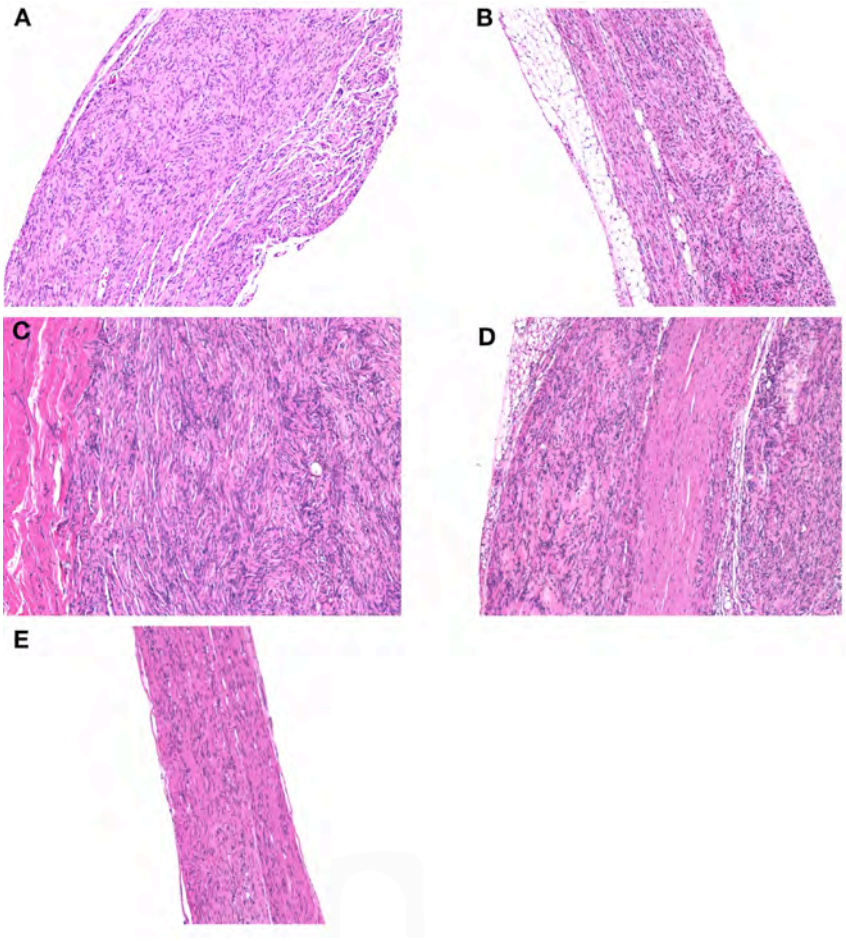


Figure 13. Injury promotes tumorigenesis following Nf2 deletion in adult mice and synergizes with activated K-Ras. **A**. The sciatic nerve of a Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$ mouse 19 weeks post-injury and 4-hydroxytamoxifen administration (10 mg/ml in DMSO at injury site) demonstrating an enlarged proximal nerve stump with tumor formation. Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *K-ras* ^{G12D} mice showed accelerated tumorigenesis following injury (**C**, 8 wks post-injury) compared either Rosa26-CreER^{T2}; *K-ras* ^{G12D} (**B**, 8 weeks post-injury) or Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$ mice (**A**, 19 weeks post-injury). Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *K-ras* ^{G12D} sham operated mice treated with 4-hydroxytamoxifen also rapidly developed tumors in the absence of injury (**D**, 8 weeks post-injury), but mice with Nf2-loss alone or K-ras activation alone did not (data not shown). **E**. A control treated nerve at 8 weeks post injury.

The *Nf2* gene in Neural Crest Patterning and Development. Dr. Giovannini and collaborators are utilizing *in vitro* and *in vivo* approaches to investigate whether the developmental stage of neural crest-derived cells (NC) may determine their susceptibility to *Nf2* loss. Pure cell populations of NC cells were obtained as an outgrowing flow of cells from E9.5 mouse embryos neural tube explants (24) and, alternatively, by fluorescence activated cell sorting (FACS) from embryos carrying a transgene allowing expression of the green fluorescent protein (GFP) specifically in neural crest cells (25). In neural tube explants from E9.5 embryos, migrating NCC appear as a homogeneous population of Sox10/p75-positive cells within 18 hours after plating (**Fig. 14A**). About 96 hours after migration, NCC cells in culture are organized in structures mimicking the organization of the peripheral nervous system (PNS): they form rounded aggregates containing glial cells (GFAP+) and neurons (Tuj1+) and are localized at a constant distance from the neural

tube explant to which they are connected by interdigitating cellular extensions. Overall, this morphological pattern is reminiscent of dorsal root ganglia (DRG) organization (**Figs. 14B, 14C**).

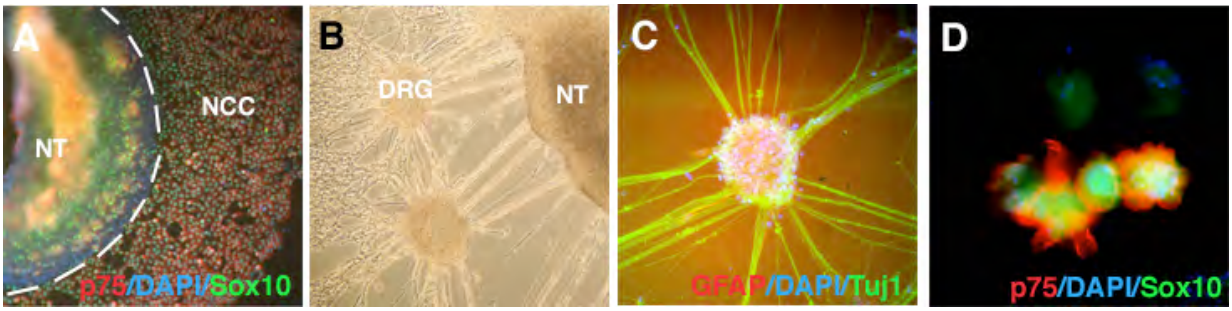


Figure 14*. NC cells from neural tube explants at embryonic day E9.5.** ***(A) *NC cells are characterized by double staining p75/Sox10. A homogeneous layer of NCC cells emanates from neural tube explants 18 hours after plating.* ***(B)*** *NC in vitro organize in structures resembling dorsal root ganglia.* ***(C)*** *Immunoflorescence staining of B shows presence of glial cells (GFAP) and nervous cells (Tuj1) cells.* ***(D)*** *Only in explants coming from HtPAcre ;$Nf2^{flox/flox}$ has been found cells with morphological characters of apoptotic cells, such as pycnotic nuclei and cell membrane blobbing. Abbreviations: NT, neural tube; NCC, neural crest cells; DRG, dorsal root ganglion-like structure.*

In E9.5 *plpGFP* transgenic mouse embryos, GFP-positive cells delaminate from the most apical region of the neural tube **(Fig. 15A).** Dr. Giovannini and collaborators were able to purify these cells by using a combination of microdissection, FACS, and culture in a defined neural crest medium (26). Remarkably, GFP^+ cells were found to behave as two different subpopulations, depending wether they derive from cephalic (brachial arches) or trunk (neural cord) regions. When treated with neuregulin (NRG) or BMP2/4, GFP^+ cells from the dissected neural cord behave as classically described for NC cells, giving rise to glial cells and neurons, respectively (**Fig. 15B**). In contrast, GFP^+ cells derived from the brachial arches, are not sensitive to these growth factors (**Fig. 15C**). Thus, NC cells in brachial arches seem to become committed earlier than NCC of the neural cord. To answer the question whether *Nf2* plays a role in NC commitment and in response to growth factors, matings have now been established to generate *Plp*GFP;$Nf2^{flox/flox}$;*HtPA*Cre embryos. Finally, the effects of *Nf2* gene loss on the expression of genes (*Sox-9, Sox-10, Id2, Ap2, c-Myc, Twist*) (27) known to be involved in the patterning of neural crest will be analyzed by whole mount *in situ* hybridization.

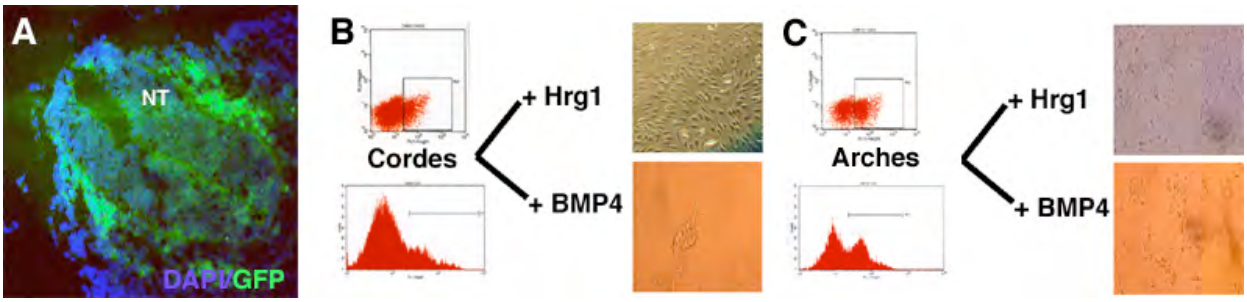


Figure 15*. plpGFP transgenic embryos express the GFP in NC.** ***(A) *GFP positive cells delaminate from apical region of neural tube.* ***(B)*** *After FACS, NC cells from truncal regions give rise to Schwann and nervous cells in response to Hrg1 and BMP4, respectively.* ***(C)*** *Differently than B, NC cells sorted from cephalic region do not give any morphologically classifiable cell type.*

To specifically induce *Nf2* gene inactivation in NCC, the *Nf2*$^{flox/flox}$ mice were crossed with two lines of transgenic mice, *Wnt1-Cre* and *HtPA-Cre*, known to efficiently induce recombination of floxed genes in the neural crest cell lineage at all axial levels of the embryo (28). In neural tube explants of *HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos, variable numbers of cells with pycnotic nuclei were observed, suggesting that loss of *Nf2* induces apoptosis in NC cells (**Fig. 14D**). As an alternate method to inactivate *Nf2* in NCC, Dr. Giovannini and collaborators inactivated the *Nf2* gene in cell cultures obtained from E9.5 *Nf2*$^{flox/flox}$ embryos and infected with an adenovirus expressing the Cre recombinase. The cultures were infected at subconfluency, split, and fixed when confluent. Besides a small increase in the speed of the cell cycle, the most remarkable phenotype in *Nf2*$^{-/-}$ NCC cultures was the maintenance of p75 and Sox10 positive cells together with the absence of differentiated cells. SCs, as NCC, are double-positive for p75 and Sox10 (24); however, *Nf2*$^{-/-}$ cells in these cultures did not show the typical SC morphology and were GFAP-negative. Therefore, based on the specific labelling and their morphology, these p75/Sox10 positive cells can be classified as NCC-like cells. Again, variable amounts of cells in apoptosis were observed. By mimicking the environmental influences that neural crest cells encounter *in vivo*, it is possible to induce *in vitro* the commitment of these cells to differentiation toward a specific lineage simply modifying the composition in growth factors of the culture medium (24). This *in vitro* approach is useful to investigate which role *Nf2* plays a role in regulating the commitment of NC cells.To analyze the effects of *Nf2* loss on neural crest cell differentiation Dr. Giovannini and collaborators switched *Nf2*$^{+/+}$ and *Nf2*$^{-/-}$ neural crest cells to defined culture media promoting differentiation toward the different NCC-derived lineages.

To define the consequences of *Nf2* loss on NCC differentiation *in vivo*, Dr. Giovannini and collaborators specifically directed *Nf2* loss to neural crest cells by crossing *Nf2*$^{flox/flox}$ mice to NCC-specific Cre transgenic mice. In particular, the consequences on peripheral nervous system (PNS) development and tumorigenes were evaluated. In addition to *P0-Cre* mice, Dr. Giovannini and collaborators have imported two lines of transgenic mice, *Wnt1-Cre* (kindly provided by A. McMahon) and *HtPA-Cre* (kindly provided by S. Dufour), known to efficiently induce recombination of floxed genes specifically in the neural crest cell lineage at all axial levels of the embryo (28). Due to the difficulty of visualizing motile cells in living vertebrate embryos, the the β-gal Cre reporter transgene *ACZL* (29) was introduced by crossing into the *Nf2* floxed strain to track the *Nf2*-mutant neural crest cells. As for *P0cre*;*Nf2*$^{flox/flox}$, Dr. Giovannini and collaborators found that *HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos exhibit an array of embryonic phenotypes that can be traced back to neural crest defaults. In contrast, *Wnt1-Cre*;*Nf2*$^{flox/flox}$ embryos die before embryonic day E9.5, similar to *Nf2*$^{-/-}$ knockout mice (17). This is probably due to Cre expression in the pre-implantatory phases of development, in a pattern similar to endogenous *Wnt1* gene expression (26). About 30% of *HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos show aberrant development at E9.5, with a dramatic reduction in size especially at the forebrain level (**Fig. 16A**).

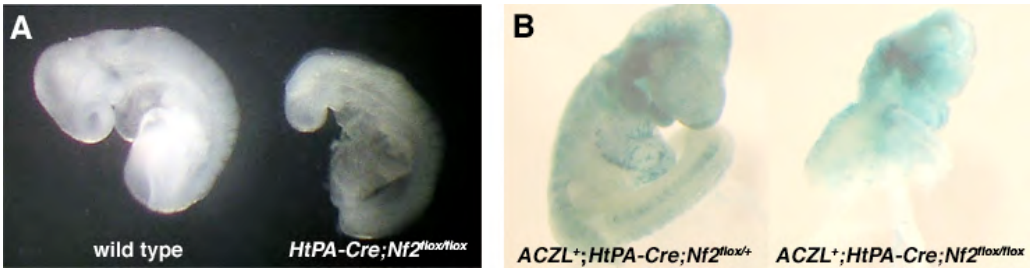


Figure 16. Loss of Nf2 in NC has dramatic effects on mouse embryonic development. **(A)** At E9.5, *HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos show a dramatic reduction in size especially at the forebrain level. **(B)** NC still survive despite *Nf2* loss and *ACZL*$^{+}$;*HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos present numerous β-gal cells.

As suggested by the neural tube explants experiments *in vitro*, the defect could be due to increased apoptosis. However, despite *Nf2* loss many NC survive, since *ACZL*$^{+}$;*HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos at E9.5 show the presence of numerous β-gal-expressing cells (**Fig. 16B**). At E18.5 some *HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos show palatoschisis, a "neural crest" phenotype , and/or a defect during circulatory system formation (**Figs. 17A, B**). Moreover, in some mutant embryos defects of cartilage formation were observed using a Alcian blue/Alizarin red staining (**Fig. 17C**). Intriguingly, about 40% of *HtPA-Cre*;*Nf2*$^{flox/flox}$ embryos that showed no apparent phenotypes died soon after birth. An extensive histological analysis is ongoing to understand the causes of perinatal death.

Figure 17*. In late embryonic stage, some HtPA-Cre;Nf2$^{flox/flox}$ embryos show default linked to NC. (A)** *Facial palatoschisis (arrow).* ***(B) *Blood vessels absence (arrow shows a head vessels missing in the mutant.* ***(C).*** *Alcian blue/Alizarin coloration shows absence of cartilaginous structures in newborn mice.*

Finally, Dr. Giovannini and collaborators are analyzing the gene-regulatory networks driving neural crest development. The extensive crossregulation of *Slug/Snail, AP-2, FoxD3, Sox10, Sox9, Id2* and *c-Myc* expression makes assigning hierarchical relationships to these factors risky. Nevertheless, they are investigating whether *Nf2* affects this molecular cascade by performing whole mount *in situ* hybridization for the above listed genes on *Nf2*$^{-/-}$ E9.5 embryos (**Figs. 18A, B**).

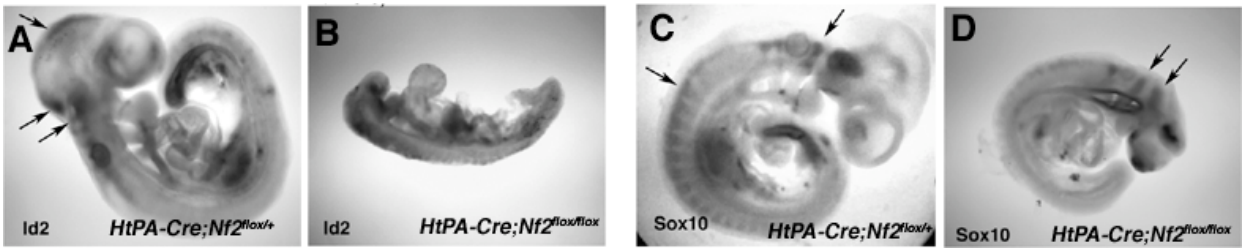


Figure 18*. Whole mount in situ hybridization for genes involved in the patterning of NC. (A and C)** *Expression patterns of Id2 and Sox10 genes in wild type E9.5 embryos.* ***(B and D) *Aberrant expression of the Id2 and Sox10 genes in HtPA-Cre;Nf2*$^{flox/flox}$ *embryos.*

Investigation of ErbB Signaling in *Nf2*$^{-/-}$ SC. The McClatchey lab previously found that primary *Nf2*$^{-/-}$ cells of several types fail to establish stable adherens junctions (AJs) and to undergo contact-dependent inhibition of proliferation (30). More recently, they discovered that Merlin can physically coordinate the process of AJ stabilization with silencing of the epidermal growth factor receptor (EGFR)(31). Their work suggests that upon cell contact, Merlin physically associates with EGFR via the PDZ-domain containing adapter NHE-RF1, and sequesters it into a

nonsignaling membrane compartment. These studies were carried out in primary fibroblasts, osteoblasts and liver epithelial cells.

EGFR (ErbB1) is the prototype of the ErbB family of tyrosine kinase receptors (ErbB1-4. SCs express multiple ErbB family members and are dependent upon ErbB signaling for proliferation and survival. Moreover, SCs display complex and exquisite coordination between ErbB receptor signaling and inter- and intracellular adhesion. The McClatchey lab initially studied primary *Nf2*$^{-/-}$ SCs isolated from the dorsal root ganglia (DRG) of 12.5 day old *Nf2*$^{lox/lox}$ embryos and infected with Ad-Cre or Ad-GFP. They found that, like other *Nf2*$^{-/-}$ cell types, *Nf2*$^{-/-}$ SCs fail to undergo contact-dependent inhibition of proliferation. Notably this occurs specifically in the presence of neuregulin1 (NRG/GGF), the major ErbB ligand for SCs; if NRG is replaced with other growth factors such as PDGF, IGF or serum, *Nf2*$^{-/-}$ SCs do undergo contact-dependent inhibition of proliferation. Importantly, treatment of *Nf2*$^{-/-}$ cells with pharmacologic EGFR inhibitors (gefitinib/Iressa, erlotinib/Tarceva) yields dramatic reversion of their morphologic phenotype, restoration of contact-inhibition, a marked reduction of active (phosphorylated) MAPK and AKT and increased levels of active phosphorylated ErbB2. This is surprising given the accepted specificity of these inhibitors for EGFR and the fact that SCs are thought to express no or very little EGFR and instead predominantly express ErbB2 and ErbB3. A major goal of the McClatchey lab over the past year has been to define the mechanistic basis of the sensitivity of *Nf2*$^{-/-}$ SCs to gefitinib/erlotinib.

Ligand-activated EGFR either homodimerizes or heterodimerizes with other ErbB family members. Dr. McClatchey's group noted that the reported NHE-RF-interacting site in the EGFR is also conserved in ErbB3 and not in ErbB2 or 4, suggesting that Merlin may also associate with and control the membrane distribution of ErbB3 and perhaps ErbB1- or ErbB3-containing heterodimers (**Fig. 19**). Initial studies revealed that NHE-RF1 readily associates with ErbB3 in *Nf2*$^{-/-}$ SCs; Dr. McClatchey's group is now testing a version of ErbB3 in which two putative NHE-RF-interacting sites have been mutated. Dr. McClatchey's group also found that *Nf2*$^{-/-}$ SCs also exhibit markedly elevated levels of pErbB3, which are reversed by gefitinib/erlotinib treatment. Together with the fact that Nrg is the major ligand for ErbB3 and that the failure of *Nf2*$^{-/-}$ SCs to undergo contact-dependent inhibition of proliferation is Nrg-dependent, these data suggest that deregulated ErbB3 signaling may be central to the overproliferation of *Nf2*$^{-/-}$ SCs. The preferred heterodimeric partner of ErbB3, which is kinase-inactive, is thought to be ErbB2, which is kinase active but cannot bind ligand. The McClatchey group noted that there is a marked increase in the levels of ErbB2:3 association in *Nf2*$^{-/-}$ SCs. This would be consistent with a model wherein Merlin normally sequesters ErbB3 into a membrane compartment from which it cannot heterodimerize with ErbB2; the McClatchey lab is actively testing this model, both in SCs and in other types of *Nf2*$^{-/-}$ cells.

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ErbB3  ERVSMCRSRSRSRSPRPRGDSAYHSQRHSLLTPVTPLSPPGLEEEDGNGYVMPDTHLRGTSSSREGTLSSVGLSSVL 1183
ErbB4  DDSCCNGTLRKPVAPHVQEDSS--TQRYSADPTVFAPERNPRGELDEEGYMTPMHDK-----PKQEYLNPVEENPFV 1156
ErbB1  -NNSTVACINRNGSCRVKEDAF--LQRYSSDPTGAVT-------EDN----IDDAFL-----PVPEYVNQSVPKRPA 1102
ErbB2  DGDLAVGVTKGLQSLSPHDLSP--LQRYSEDPTLPLP-------PETDGYVAPLACS-----PQPEYVNQ--PEVRP 1148
```

Figure 19. Potential internal PDZ-domain binding sites in ErbB receptors*. The two internal binding sites for EBP50 that have been previously identified in ErbB1 are indicated in* ***blue*** *(55). The sequence is DSFL in human ErbB1. We note that similar PDZ-binding consensus sequences are present in the same region of ErbB3 (****red****) but not ErbB2 or ErbB4.*

ErbB levels in *Nf2*$^{-/-}$ Schwann cell xenografts. Both Drs. McClatchey and Giovannini have found that after a few passages *Nf2*$^{-/-}$ SCs readily form tumors when injected subcutaneously in to nude mice. After establishing cell lines from several such subcutaneous 'schwannomas', Dr. McClatchey's group examined the levels of expression of each ErbB family member by RT-PCR and compared to that of the starting primary SCs. Surprisingly, after 'passage' through a subcutaneous xenograft, *Nf2*$^{-/-}$ SCs exhibited a change in the ErbB expression program, including increased ErbB1 mRNA levels and decreased ErbB3 and ErbB4 mRNA levels **(Fig. 20).** Perhaps this reflects the adaptation of these cells to the Nrg-deficient subcutaneous environment given the dependency of cultured primary *Nf2*$^{-/-}$ SCs upon Nrg. Regardless of the mechanism, this observation has important implications for preclinical studies involving SC-derived xenografts.

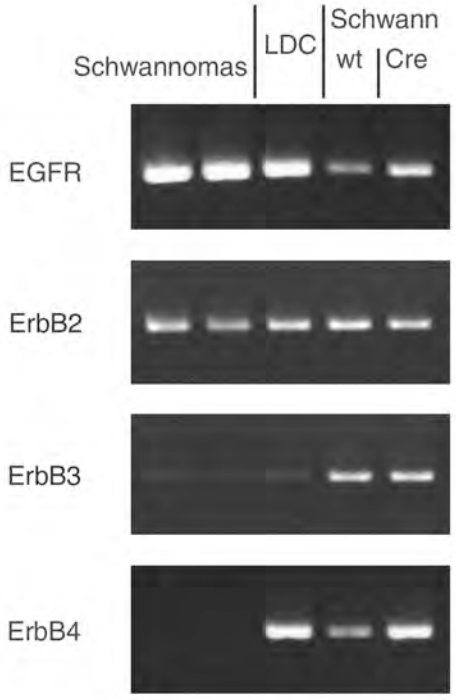

***Figure 20*. Altered expression of ErbB family members in *Nf2*$^{-/-}$ Schwann cells (SCs) grown as subcutaneous xenografts.** *Primary Nf2*$^{-/-}$ *SCs (right) are injected subcutaneously and readily form subcutaneous tumors ('schwannomas', left). Wild-type SCs do not for subcutaneous tumors. RT-PCR reveals that EGFR mRNA levels are markedly elevated and those of ErbB3 and ErbB4 downregulated in cells derived from Nf2*$^{-/-}$ *SC xenografts. ErbB2 mRNA levels remain unchanged. LDC, liver-derived cells.*

Merlin-Mediated Regulation of ErbB Receptor Distribution and Signaling Across Multiple Cell Types. Central to understanding the gefitinib/erlotinib sensitivity of *Nf2*$^{-/-}$ SCs is the further delineation of the mechanism whereby Merlin regulates the surface availability of EGFR (ErbB1) and ErbB3 and consequent signaling in any cell type. Increasing evidence suggests that the function of Merlin is context-dependent; one way that this could occur is through the differential expression and heterodimerization of different ErbB family members. The McClatchey group is actively investigating the consequences of *Nf2*-deficiency on ErbB receptor distribution and signaling across a number of different cell types *in vitro* and *in vivo*. Their studies thus far reveal that *Nf2*-deficiency yields overproliferation of many epithelial tissues *in vivo*, including the liver, skin, intestine and mammary gland and that this overproliferation is associated with altered ErbB receptor levels and/or signaling in all cases (**Fig. 21;** and data not shown). Ongoing molecular studies aim to detail ErbB surface availability, solubility, dimerization and signaling in both tissues and cell cultures established from each of these models; the sensitivity of *Nf2*$^{-/-}$ cells of each type to pharmacologic inhibition of EGFR and other ErbBs will also be determined. Comparison of these results to the ongoing studies of *Nf2*$^{-/-}$

Schwann cells will facilitate an understanding of the context-dependency of Merlin function, which has important implications for considering human NF2-associated tumors beyond schwannomas.

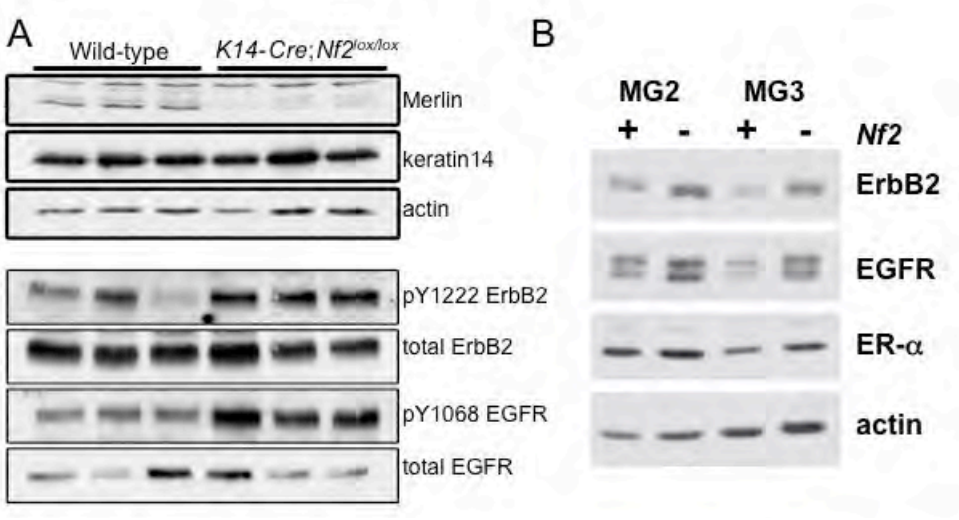

Figure 21. Altered ErbB levels and/or signaling in $Nf2^{-/-}$ tissues. A. *Epidermal tissue from K14-Cre;$Nf2^{lox/lox}$ mice displays elevated levels of active, phosphorylated ErbB2 and ErbB1 (EGFR). Samples from three different mice of each genotype are shown.* ***B.*** *Mammary gland tissue from MMTV-Cre;$Nf2^{lox/lox}$ and control mice reveals elevated levels of total ErbB2 and EGFR.*

Analysis of Mammalian Expanded Localization.Dr. McClatchey's lab has cloned the mammalian version of the Merlin-related tumor suppressor Expanded (Frmd6) and continues to characterize its function. They generated a polyclonal antibody directed against Expanded and found that Expanded colocalizes with Merlin at apical junctional structures. This is markedly similar to the reported localization of *Drosophila* Expanded in epithelial tissues (32). Unlike *Drosophila* Expanded, however, the McClatchey group has been unable to detect a physical association between mammalian Expanded and Merlin. Moreover, Expanded localization is apparently not dependent upon the presence of Merlin as the staining pattern for Expanded is identical in WT and $Nf2^{-/-}$ cells. Biotinylation and membrane fractionation experiments suggest that Expanded associates with membrane proteins. As in other ERM proteins, this membrane associating activity resides in the N-terminal FERM domain, as the N-terminal half of Expanded, when expressed alone, exhibits membrane localization, while the C-terminal half does not. Notably, in screening a panel of candidate Expanded interactors, the McClatchey lab found that like Merlin, Expanded co-immunoprecipitates with E-cadherin in an adhesion-dependent manner (**Fig. 22, next page**). However, Expanded can localize to the membrane regardless of the status of cell adhesions, suggesting that membrane receptors other than E-cadherin are responsible for recruiting Expanded to the membrane. Importantly, however, unlike Merlin, Expanded does not associate with EGFR and EGF stimulation does not grossly alter Expanded localization. These data suggest that Expanded, like Merlin, may function to coordinate cell adhesion receptors with other, perhaps mitogenic receptors at the membrane. The McClatchey lab is actively seeking the identity of other Expanded-associated receptors in SCs and other cell types.

Investigating the Role of Mammalian Expanded and Merlin in Hippo/Warts Signaling. Recent studies in *Drosophila* suggests that Expanded and Merlin, together, function upstream of the Hippo/Salvador/Warts/Yki pathway (33). Dr. McClatchey's lab has begun to determine whether mammalian Expanded and Merlin also function in the mammalian counterpart of the Hippo/Warts/Yki pathway (Mst/Lats/YAP). The *Drosophila* studies demonstrated that overexpression of fly Expanded and Merlin activates the Hippo pathway as measured by phosphorylation of Warts. Although initial studies in Dr. McClatchey's lab did not reveal major changes in the phosphorylation of mammalian Mst2 (Hippo) or Lats1 (Warts) in cells overexpressing Expanded, Merlin or both proteins, more sophisticated studies that examined the context-dependent regulation of this pathway were more intriguing. Notably, their results suggest that Mst/Lats can be regulated by either cell adhesion or by EGFR signaling but *only if* Merlin is present. This raises the possibility that Merlin acts as a 'clutch', facilitating engagement of upstream receptors with the Hippo pathway. Similarly, Expanded may allow the engagement of the Hippo pathway in response to other membrane receptors. The McClatchey lab is actively pursuing this idea.

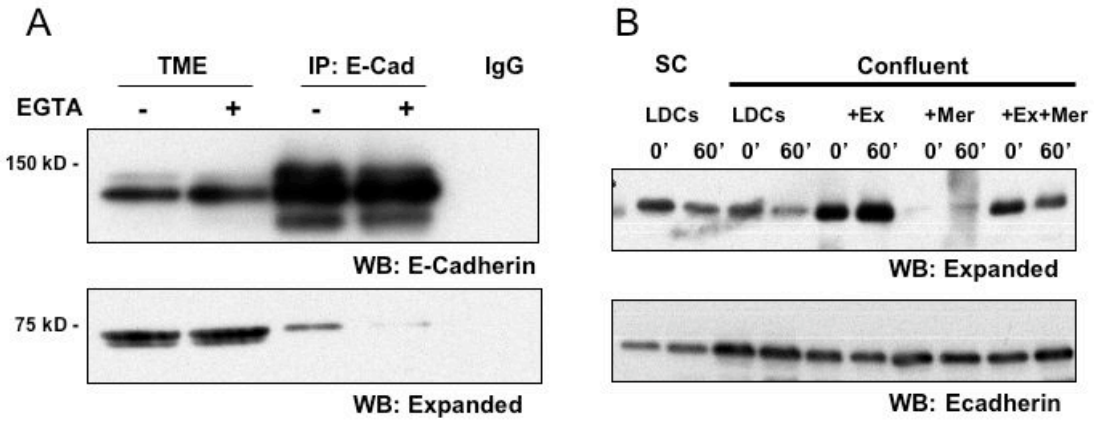

Figure 22*. *Membrane association of mammalian Expanded. A. *Co-immunoprecipitation (IP) of mammalian Expanded with E-cadherin occurs in the membranes of confluent cells; acute disruption of cell:cell adhesion with EGTA treatment yields loss of Expanded:E-cadherin association.* ***B.*** *In contrast, cell surface biotinylation reveals that Expanded associates with biotinylated surface proteins under both subconfluent (SC) and confluent conditions. TME, total membrane extract. LDCs, liver derived epithelial cells.*

Generation of an *Expanded* Mutant Allele. To generate a valuable tool for delineating the molecular function of mammalian Expanded and to investigate the function of Expanded in tumorigenesis, Dr. McClatchey's lab has designed and engineered a conditional mutant allele of mouse *expanded*. This *expanded* mutant allele features loxP sites flanking exon 2 and a promoterless β-geo cassette flanked by frt sites that is designed to report endogenous *expanded* expression *in vivo*. Expression of the Cre- recombinase will yield excision of exon 2, resulting in an immediate frame shift and a presumably null allele; Flp-mediated recombination will remove the β-geo cassette. The engineered targeting construct was electroporated into J1 and W4 embryonic stem (ES) cells. A total of 4 correctly targeted ES cell clones were identified out of more than 500 drug-resistant clones screened by Southern blot analysis and PCR in two separate

experiments. The first two clones were injected into blastocysts and transplanted into recipient female mice yielding 11 chimeric progeny all from one clone. Two of the chimeras (both male) yielded germline progeny; however, all of more than 40 F1 progeny carried the WT *expanded* allele. This raises the possibility that the *expanded* mutant allele functions as a severely hypomorphic, null or neomorphic allele that interferes with spermatogenesis. The McClatchey lab is now injecting independently derived correctly targeted ES cell clones into blastocysts; they are also infecting correctly targeted ES cells with Ad-Flp to remove the resistance cassette and leave only two intronic loxP sites in the *expanded* locus, presumably restoring WT function.

Other Contributions

A previous DAMD award to our group (DAMD17-02-1-0638) supported a workshop entitled *"Barriers and Solutions in the Use of Mouse Models to Develop Therapeutic Strategies for NF1 and NF2-Associated Tumors"* that Dr. Shannon organized with Drs. David Gutmann (Washington U.) and Kim Hunter-Schaedle (Children's Tumor Foundation). The attendees identified a number of practical strategies to achieve the goal of using mouse models of NF-associated tumors to facilitate the goal of bringing new treatments to patients. A review article summarizing general principles discussed at this meeting and implications for developing and evaluating new treatments for tumors in individuals with NF1 and NF2 disease was published last year (34). In an important recent development, the Children's Tumor Foundation announced the formation to a Preclinical Drug Discovery Initiative (DDI) that will provide $250,000 per year for three years to five research groups that will collaborate closely to test novel treatments in mouse models of NF1 and NF2 disease. This important effort for NF patients and their families is only possible because of mouse models that were developed though this consortium effort.

KEY RESEARCH ACCOMPLISHMENTS

(a) The investigators continue to extensively share expertise and reagents to pursue common research goals.

(b) The NF Modeling Group is participating in the activities of the MMHCC and is contributing to achieving the goals of this national cancer research initiative.

(c) We found that the loss of contact-dependent inhibition or proliferation exhibited by *Nf2*-deficient Schwann cells , is Nrg-dependent, is associated with increased activation of ErbB3 and is blocked by both Gefitinib and Tarceva. We also found that Merlin can associate with ErbB3 and that Tarceva treatment restores low levels of active ErbB3 to *Nf2*-/- SCs.

(d) We found that the subcutaneous growth of $Nf2^{-/-}$ SCs is associated with an altered profile of ErbB expression.

(e) We have found that alterations in the levels of and signaling from ErbB receptors occurs in several types of epithelial tissues.

(f) We found that Expanded associates with E-cadherin in an adhesion-dependent manner and may play a role in coordinating E-cadherin mediated cell:cell contact with regulated signaling by other membrane receptors.

(g) We have refined our mouse models of neurofibromas through the isolation of precursor cells that can be genetically manipulated and introduced into the same mouse to form tumors.

(h) MPNST-derived cells are being tested to discover novel molecules that may interrupt their growth in collaboration with the chemical screening facility at UTSW Department of Biochemistry.

(i) We have developed a robust model for NF1-associated optic glioma. These mice are now being placed in preclinical trials for prevention and tumor arrest therapies.

(j) We have developed a robust model for NF1-associated astrocytoma. We have identified the cells that give rise to the tumor, purified them, and are in the process of screening for chemical compounds that inhibit growth.

(k) We have developed a robust model for dermal neurofibromas that completely replicate the human tumors. These mice will serve as substrates for preclinical testing.

(l) Studies performed in mouse neurofibroma led directly to a phase II clinical trials using imanitib to treat plexiform neurofibromas. The rationale for this novel trial is based on the ability to regress plexiform neurofibromas in our mice by inhibiting mast cell function.

(m) A new method was developed for isolating mitotically active SCs that can also be used for the purification/enrichment of early NCC-derived cells.

(n) A novel strain of drug-inducible Cre mice was constructed and is being harnessed to develop models of adult onset tumors by deleting the *Nf1* and *Nf2* genes in specific tissues at defined time points.

(o) We have begun characterizing the role of *Nf2* in wound healing and the role of injury in tumor initiation.

(p) We have observed cooperative tumorigenic effects of *Nf2*-loss and oncogenic *Kras* expression in a model of ependymona and in schwannoma formation.

(q) Studies of MEFs and bone marrow cells support the idea that p120GAP functions both as a negative regulator of Ras signaling and to promote growth.

(r) The biochemical consequences of *Nf1* inactivation in primary cells was investigated by Western blotting and by phospho-FACS analysis of bone marrow cells from *Mx1-Cre* *Nf1* $^{flox/flox}$ mice.

(s) RIM was used to generate aggressive leukemias, to identify genes and pathways that cooperate with *Nf1* inactivation in tumorigenesis, and to investigate mechanisms of response and resistance to MEK

(t) Strains of mutant mice have been shared widely with the NF research community (see list below in Reportable Outcomes) and comprise an integral resource of the Preclinical Drug Discovery Initiative of the Children's Tumor Foundation. Through these collaborative experiments, the scientific value of this consortium has extended well beyond the studies being pursued in the participant's laboratories.

REPORTABLE OUTCOMES

(a) Research Articles and Reviews from Previous Consortium Awards (2000 – 2005)

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(b) Model Development and Distribution to the Research Community

Studies conducted to date have established a number of novel models of NF1 and NF2-associated tumors and have generated several new strains of mice. *Nf1* and *Nf2* mutant mice have been deposited in the MMHCC Repository where they are readily available to the research community. In addition, the participants in this Consortium have provided strains directly to the investigators listed below.

Karlene Reilly (National Cancer Institute)
Jeffrey DeClue (National Cancer Institute)
Jonathan Epstein (University of Pennsylvania)

D. Wade Clapp (Indiana University)
David Guttmann (Washington University)
David Largaespada (University of Minnesota)
Jeffrey Lawrence (UCSF)
Alcino Silva (UCLA)
Gerard Karsenty (Baylor)
Shaojun Tang (UC Irvine)
Shalom Avraham (Beth Israel)
James Bieker (Mount Sinai, New York)
Abhijit Guha (Labatt Brain Tumor Research Center, Tornoto)
Andreas Kurtz, (Harvard)
Jim Gussella (Harvard)
Dan Haber (Harvard)
Antonio Chiocca (Harvard)
Isidro Sanchez-Garcia (IBMCC)
Victor Tybulewicz (National Institute for Medical Research, London)
Lindsay Hinck (UC Santa Cruz)
Keqiang Ye (Emory University School of Medicine)
Lynda Chin (Dana Farber Cancer Institute)
Joseph Testa (Fox Chase Cancer Center)
Nancy Ratner (U. of Cincinnati)
Stefan Mundlos (U. of Berlin)
Juha Peltonen (U. of Helsinki, Finland)
Warren Pear (University of Pennsylvania)
David Beebe (Washington University)
Filippo Giancotti (MSKCC, New York)
Joe Kissil (Wistar Institute, Philadelphia)
Long Sheng Chang (Ohio State University, Columbus)
Cristina Fernandez Valle (University of Central Florida, Orlando)
Silvia Espejel (University of California, San Francisco)
Karen Cichowski, (Harvard)
Sean J. Morrison, (University of Michigan)
John J. Ryan, (Virginia Commonwealth University)
Isa Hussaini, (University of Virginia)
William Pu, (Children's Hospital, Boston)
Filippo G. Giancotti (Sloan-Kettering Institute for Cancer Research)
David Wilkes (Cornell University Medical College)
Ivan Radovanovic (Geneva)
Brian Weiss (University of Cincinnati)
Arturo Alvarez-Buylla (University of California, San Francisco)
Jonathan Chernoff, (Fox Chase Cancer Center)
Laurent Elefteriou (UT San Antonio)
David Kaplan (Toronto)
Hong Wu (UCLA)
Ugur Ozerdem (La Jolla Insitiute)
David Ingram (Indiana U)

Takayuki Harada (Tokyo)
Alison Lloyd (London, UK)
Junichi Sadoshima (New Jersey Medical School and Johns Hopkins)
Susan Lindquist (Whitehead Institute at MIT)
Richard Bram (Mayo Clinic Rochester)
Jean Nakamura (University of California, San Francisco)

(c) Employment and Research Opportunities

This award has provided salary support for technical personnel and for trainees in each of participating labs.

CONCLUSIONS

During the seventh year of its existence, this consortium made additional progress in generating and characterizing mouse models of NF1 and NF2-associated tumors. Novel strains have been developed and reported, innovative strategies were deployed to make optimal use of these resources, and our recent research has provided a number of new insights regarding how the *Nf1* and *Nf2* gene products regulate cellular processes such as survival, adhesion, proliferation, wound healing, self-renewal, and signal transduction. We note the recent Preclinical Drug Discovery Initiative of the Children's Tumor Foundation as a remarkable example of how strains of mice engineered by this consortium are being utilized in innovative ways to advance NF research and facilitate the testing of new treatments for the complications of NF1 and NF2. The investigators continue to collaborate closely and have shared expertise and reagents extensively. The overall goal of the next fund year is to continue working to achieve the Technical Objectives of this project.

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